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ISOMERIZATION OF 11-*cis* VITAMIN A IN VIVO[1]

D. W. STAINER,[2] T. K. MURRAY, AND J. A. CAMPBELL

Abstract

Single oral doses of all-*trans* and 11-*cis* vitamin A acetate were given to young, vitamin A deficient rats and the proportion of *cis* isomer in the intestinal tract and liver measured. Some conversion of 11-*cis* to all-*trans* occurred in the stomach and intestine, and a mixture of the two isomers was absorbed and stored in the liver. The high proportion of *cis* isomer found in the liver stores 5 hours after a dose of 11-*cis* vitamin A disappeared completely in 23 days. Oral doses of both all-*trans* and 11-*cis* vitamin A produced greater liver stores than the same doses given subcutaneously. The relative biological potency of the 11-*cis* isomer was the same by either route, which indicated that the low potency of this isomer was not due only to poor absorption from the intestine.

Although the 11-*cis* isomer of vitamin A plays an essential role in scotopic vision it has, in common with other *cis* isomers, a low biological potency when measured by growth or liver storage methods (1). It is not normally found in any mammalian tissue other than the pigment epithelium of the retina (2). Dowling and Wald presented evidence (3) that injected 11-*cis* vitamin A was transformed to the all-*trans* form before entering the rhodopsin cycle and Plack (4) found that the 11-*cis* isomer was absorbed after oral administration but quickly disappeared from the liver, apparently by conversion to the all-*trans* form. Earlier work in this laboratory (5) with neovitamin A (13-*cis*) showed that isomerization took place in the intestinal tract as well as in the liver. It is the purpose of this paper to describe the behavior of 11-*cis* vitamin A in the intestinal tract and liver and attempt to relate the results to the low biological potency of this isomer.

Procedure and Results

Isomer Content of the Gastrointestinal Tract and Liver following Oral Doses of All-trans or 11-cis Vitamin A

Young, male, vitamin A deficient rats of an inbred Wistar strain were divided into two groups of 10 animals and were dosed orally with all-*trans* or 11-*cis** vitamin A acetate. In the first experiment, one group received 2820 units of 11-*cis* vitamin A, the other 2370 units of all-*trans* vitamin A, determined by the antimony trichloride reaction. In experiment 2, the doses con-

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tained 2800 and 2200 units respectively. In all cases, the total dose was contained in 0.1 ml of corn oil and was administered with a tuberculin syringe and blunted needle. The animals were killed by decapitation 5 hours after dosing and the gastrointestinal tract and liver removed and analyzed for total vitamin A and *cis* isomer content by the techniques previously described (5). Table I shows the results obtained. After a dose of 11-*cis* vitamin A, the

TABLE I

Vitamin A and *cis* isomer content of the gastrointestinal tract and liver 5 hours after an oral dose of 11-*cis* or all-*trans* vitamin A

		11-*cis* vitamin A*		All-*trans* vitamin A*	
Tissue	Experiment	% of dose	% *cis* isomer	% of dose	% *cis* isomer
Stomach	1	5	62	2	33
	2	8	67	9	39
Intestinal contents	1	4	43	3	24
	2	2	53	3	23
Intestine	1	2	43	2	12
	2	1	42	2	12
Liver	1	5	24	19	3
	2	4	36	14	6
Caecum	1	4	58	1	58
	2	2	66	3	50

*The dose of 11-*cis* was 2820 units in experiment 1 and 2800 in experiment 2. All-*trans* dose was 2370 units in experiment 1 and 2200 in experiment 2. Both determined by $SbCl_3$ reaction.

vitamin in the stomach comprised some 60% *cis* isomer, that in the intestinal tract 43–53%, and that in the intestine 43%. The vitamin found in the liver 5 hours after an oral dose contained only 24–36% *cis* isomer. When all-*trans* vitamin A was given orally the *cis* isomer content of the stomach, intestinal contents, intestine, and liver was 33–39%, 23–24%, 12%, and 3–6% respectively. The vitamin in the caeca of both groups of animals contained more than 50% *cis* isomer. The all-*trans* dose produced three to four times greater liver stores than did the 11-*cis* but there was no difference in the total vitamin A found in the digestive tract.

Vitamin A Depletion following a Dose of 11-cis or All-trans Vitamin A

Vitamin A deficient rats, similar to those used in the previous study, were dosed subcutaneously with 1590 units of all-*trans* vitamin A acetate or 1505 units of 11-*cis* vitamin A acetate, both dispersed in water with the aid of Tween 80. After periods of 2, 9, 23, and 37 days, eight animals from each group were killed by decapitation and the livers analyzed for total vitamin A and *cis* isomer content. Results are shown in Table II. Total vitamin A content of the liver increased up to 9 days and then declined steadily. When 11-*cis* vitamin A was administered, liver vitamin A contained 20% *cis* isomer after 2 days, 6.4% after 9 days, and was undetectable thereafter. The liver stores of rats given all-*trans* vitamin A did not at any time contain appreciable amounts of *cis* isomer.

TABLE II

Vitamin A depletion following a subcutaneous dose of 11-*cis* or all-*trans* vitamin A

Tissue	Dose	Days after dosing: 2 — Vit. A units*	2 — % *cis* isomer	9 — Vit. A units	9 — % *cis* isomer	23 — Vit. A units	23 — % *cis* isomer	37 — Vit. A units	37 — % *cis* isomer
Liver	1590 units* all-*trans*	655	1.7	770	0.6	452	1.5	297	N.D.
Liver	1505 units 11-*cis*	227	20.0	237	6.4	63	N.D.	37	N.D.

*Determined by $SbCl_3$ reaction.
N.D. Not detectable.

Liver Storage of Vitamin A after Oral and Subcutaneous Doses of All-trans and 11-cis Vitamin A

Young male rats were divided into four groups of 10 and were given a single dose, orally or subcutaneously, of all-*trans* or 11-*cis* vitamin A acetate. The vitamin was dispersed in water with the aid of Tween 80 and the potency of the doses was determined by the antimony trichloride method. Two similar experiments were completed; in one the animals were killed 2 days after dosing, in the other 8 days after dosing when the stores from the injected dose had been found to be near their peak. The total vitamin A and *cis* isomer content of the livers is shown in Table III.

TABLE III

Liver storage of vitamin A after oral and subcutaneous doses of all-*trans* and 11-*cis* vitamin A

Dose	Days after dose	Liver stores (% of dose)	% *cis* isomer
Experiment 1			
1590 units all-*trans* orally	2	50.5	0.7
1590 units all-*trans* subcutaneously	2	41.2	1.7
1505 units 11-*cis* orally	2	18.7	9.4
1505 units 11-*cis* subcutaneously	2	15.1	20.0
Experiment 2			
1900 units all-*trans* orally	8	61.4	1.8
1900 units all-*trans* subcutaneously	8	57.4	1.3
1957 units 11-*cis* orally	8	24.2	14.0
1957 units 11-*cis* subcutaneously	8	20.4	16.5

In experiment 1, oral dosing produced significantly greater liver stores than did the subcutaneous route for both isomers. In the second experiment the differences, though similar to those in experiment 1, were not significant. Subcutaneous injection of the 11-*cis* isomer appeared to result in a higher proportion of the *cis* isomer in the liver stores than did oral dosing. By either route of administration the liver stores produced by the 11-*cis* vitamin A dose amounted to 36–39% of those produced by the all-*trans* dose.

Discussion

When 11-*cis* vitamin A was given orally to rats there was considerable conversion to the all-*trans* form in the stomach and intestine. Nevertheless, the

cis isomer was absorbed and, 5 hours after dosing, some 30% of the vitamin stored in the liver was in this form. The total amount of vitamin A stored was one-quarter to one-third that stored from a dose of all-*trans* vitamin A, but the amount remaining in the intestinal tract was the same for both isomers. There was, therefore, no evidence that the smaller liver stores were the result of poorer or slower absorption of the 11-*cis* isomer. These results are similar to those reported earlier (5) for neovitamin A, except that a larger proportion of the latter isomer was converted to all-*trans* in the intestinal tract and more was stored in the liver.

The ability of the liver to convert *cis* isomers of vitamin A to all-*trans* has been demonstrated here with the 11-*cis* isomer as it was earlier with neovitamin A (5). When 11-*cis* vitamin A was injected subcutaneously, the *cis* isomer content of the vitamin stored in the liver was 20% 2 days after dosing, 6% after 9 days, and 0 after 23 days. The greatest decrease in *cis* isomer content occurred during the first 9 days when the total liver stores of the vitamin were on the increase. There seems little doubt that the decrease in *cis* isomer content represents conversion to all-*trans* and not preferential removal of the *cis* isomer from the liver. Plack (4), who measured the *cis* isomer content of liver for 7 days after dosing, reached the same conclusion.

Oral administration of either all-*trans* or 11-*cis* vitamin A resulted in larger liver stores than did subcutaneous injection but the storage of the 11-*cis* dose, relative to that of all-*trans*, was similar for both routes of administration. It should be noted that storage of the 11-*cis* isomer was greater (36–39% that of all-*trans*) than its reported potency would have suggested.

It can be concluded from these experiments that the low potency of the 11-*cis* isomer was not due to the inability of the intestine to absorb the *cis* isomer. Considerable all-*trans* was formed in the stomach, but a mixture of the two isomers was absorbed. Nor could it be shown that more of an 11-*cis* dose reached the caecum, i.e. was not absorbed. Furthermore, the 11-*cis* vitamin A had the same biological potency whether administered subcutaneously or orally. It has therefore been shown that a factor other than intestinal absorption is responsible for the low biological potency of the 11-*cis* isomer of vitamin A.

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COMBINED ESTIMATES IN COLORIMETRIC DETERMINATIONS WITH HIGH VARIABILITY[1]

P. A. ANASTASSIADIS

Abstract

In a number of colorimetric determinations used in biochemistry, the absorbances of replicates are subject to variations. The resulting variability may be time-to-time variability, measured by the intervariance, or variability of simultaneous replicates, measured by the intravariance. In such determinations two estimates of the unknown may be derived according to the standards used, one based on the absorbance of simultaneous standards, a second based on the mean absorbance of all available standards. If intravariance does not exist, the first estimate is the more precise. If intervariance does not exist, the second estimate is the more precise. If both intravariance and intervariance exist, a weighted mean of two estimates, one based on the absorbances of simultaneous standards and a second based on the mean absorbance of all the available non-simultaneous standards, is a value with an increased precision. A method for calculating this value is given.

Introduction

In colorimetry the deduction of the concentration of a substance in an unknown sample from its light absorbance is usually based on absorbance of standards (samples containing known amounts of the substance to be determined). Generally, the absorbance has its mean proportional to the concentration (the concentration – mean absorbance relationship follows Lambert–Beer law). In order to simplify the subject we will assume that this proportionality holds. Then a proportionality coefficient between concentration and absorbance of the standard(s) is used for the derivation of the concentration of the unknown from its absorbance.

In determinations exhibiting variability the estimation of the concentration of the unknown is based on the absorbance of standards treated on the same occasion with the unknown (simultaneous standards). The possibility is discussed that the precision of the estimate may be improved if the absorbances of standards treated on other occasions are also taken under consideration. Obviously, the precision of the estimate may be improved by an increase of the numbers of simultaneous standards. This approach, however, in most biochemical analyses is unpractical because a large number of simultaneous replicates of standard is either not feasible or undesirable. As a rule, only one or few standards may be treated simultaneously with the unknown, whereas the absorbances of many standards used on other occasions in the same laboratory, very often by the same analyst, are usually available for possible use. A combined estimate may therefore be calculated.

In the determination of hexosamine in tissues (1, 2), combined estimates were calculated as described here.

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Calculation of the Combined Estimate

Let σ_i^2 be the variance of a replicate within each determination (intravariance) and σ_0^2 be the variance of a replicate between determinations (intervariance). In an analytical procedure in which intravariance does not exist and the intervariance is sizeable, the proportionality coefficient calculated from a simultaneous standard is satisfactory. In an analytical procedure in which intravariance is sizeable, but intervariance does not exist, the proper proportionality coefficient would be the mean of the individual proportionality coefficients calculated from all the available standards, non-simultaneous and simultaneous. In an analytical procedure in which both intravariance and intervariance are sizeable two proportionality coefficients may be used for the determination of the concentration of the unknown.

Let K_s be the proportionality coefficient between concentration and absorbance calculated from simultaneous standards and K_0 be the proportionality coefficient calculated from all the available non-simultaneous standards. Let V_s be the estimate of the unknown derived from K_s, and V_0 be the estimate derived from K_0. Finally, let V_w be a weighted average of two original estimates, V_s and V_0. V_w will have the maximum precision (the minimum variance) when the weights assigned to each original estimate are inversely proportional to the variance of the estimate.

It may be shown that V_w is approximately equal to

$$[1] \qquad V_w \simeq \frac{K_0^2\left(\sigma_0^2+\frac{\sigma_0^2}{n_0'}+\frac{\sigma_i^2}{n_u}+\frac{\sigma_i^2}{n_0}\right)V_s+K_s^2\left(\frac{\sigma_i^2}{n_u}+\frac{\sigma_i^2}{n_s}\right)V_0}{K_0^2\left(\sigma_0^2+\frac{\sigma_0^2}{n_0'}+\frac{\sigma_i^2}{n_u}+\frac{\sigma_i^2}{n_0}\right)+K_s^2\left(\frac{\sigma_i^2}{n_u}+\frac{\sigma_i^2}{n_s}\right)},$$

where n_u is the number of replicates (simultaneous) of the unknown, n_s the number of simultaneous replicates of standards, n_0' the number of occasions on which non-simultaneous standards were treated, and n_0 the number of non-simultaneous standards.

In practice, a large number of recorded absorbances of standards analyzed on different occasions are usually available. Then [1] becomes

$$[2] \qquad V_w' \simeq \frac{K_0^2\left(\sigma_0^2+\frac{\sigma_i^2}{n_u}\right)V_s+K_s^2\left(\frac{\sigma_i^2}{n_u}+\frac{\sigma_i^2}{n_s}\right)V_0}{K_0^2\left(\sigma_0^2+\frac{\sigma_i^2}{n_u}\right)+K_s^2\left(\frac{\sigma_i^2}{n_u}+\frac{\sigma_i^2}{n_s}\right)}.$$

The use of the expression [2] is facilitated when written in the form [3] and tables may be constructed giving the value of C_0.

$$[3] \qquad V_w' \simeq (1-C_0)\,V_s+C_0\,V_0.$$

Table I gives values of C_0 for a few values of the ratios σ_i^2/σ_0^2 and K_s^2/K_0^2 and for one and two simultaneous standards and replicates of the unknown.

The V_w' value has the following variance

[4] $$\text{var}\,(V_w') = \frac{K_s^2 K_0^2\left(\frac{\sigma_i^2}{n_u}+\frac{\sigma_i^2}{n_s}\right)\left(\sigma_0^2+\frac{\sigma_i^2}{n_u}\right)}{K_s^2\left(\frac{\sigma_i^2}{n_u}+\frac{\sigma_i^2}{n_s}\right)+K_0^2\left(\sigma_0^2+\frac{\sigma_i^2}{n_u}\right)}.$$

TABLE I

Values of $[C_0]$ for some values of the ratios $[\sigma_i^2/\sigma_0^2]$ and $[K_s^2/K_0^2]$ and for one and two simultaneous standards $[n_s]$ and replicates of the unknown $[n_u]$, when a large number of non-simultaneous standards are available

n_s	n_u	$\frac{k_s^2}{k_0^2}$	Values of $[\sigma_i^2/\sigma_0^2]$				
			0.2	0.6	1.0	2	6
1	1	0.8	0.211	0.375	0.444	0.516	0.578
		0.9	0.231	0.403	0.474	0.546	0.607
		1.1	0.268	0.456	0.524	0.595	0.654
		1.2	0.286	0.474	0.546	0.615	0.673
	2	0.8	0.179	0.356	0.444	0.546	0.643
		0.9	0.197	0.384	0.474	0.575	0.669
		1.1	0.231	0.432	0.524	0.623	0.712
		1.2	0.247	0.454	0.546	0.643	0.730
2	1	0.8	0.167	0.310	0.375	0.444	0.507
		0.9	0.184	0.336	0.403	0.474	0.536
		1.1	0.216	0.382	0.452	0.524	0.586
		1.2	0.231	0.403	0.474	0.546	0.607
	2	0.8	0.127	0.278	0.348	0.444	0.546
		0.9	0.141	0.294	0.375	0.474	0.575
		1.1	0.167	0.337	0.423	0.524	0.623
		1.2	0.179	0.356	0.444	0.546	0.643

Estimates of σ_i^2 and σ_0^2 may be obtained through the analysis of variance of absorbances of replicates of unknown and (or) standards. The absorbances of standards are, however, more suitable because their concentrations are the same in analyses made on different occasions.

Example.—On 16 occasions different unknown samples were analyzed for their content of substance A by a colorimetric method. The determinations were carried out in duplicates. On each occasion duplicates of standards, having a concentration 4 μg of A per ml, were treated simultaneously with the unknown. The absorbances obtained for unknowns and standards are given in Table II. The analysis of variance for the absorbances of standards gave the following values for intravariance and intervariance: $s_i^2=0.000731$, $s_0^2=0.000862$. Then $s_i^2/s_0^2=0.8485$. Table II gives further the estimates V_s, V_0, and V_w' of the unknowns. The V_w' estimates derived from the expression [2] (Table I). (The V_w estimates derived from the expression [1] are only slightly different.) It will be noticed that quite often V_w' estimates differ substantially from the usual V_s estimate, although the value of the ratio s_i^2/s_0^2 is moderate.

The expected variances of V_s, V_0, and V_w' for an unknown having a concentration 4 μg/ml are as follows:

$$\text{var}\,(V_s) = K^2\left(\frac{s_i^2}{2}+\frac{s_i^2}{2}\right) = 0.1747,$$

$$\text{var } (V_0) = K^2\left(s_3^2+\frac{s_i^2}{2}\right) = 0.2935,$$

$$\text{var } (V_w') = K^2\frac{\left(\frac{s_i^2}{2}+\frac{s_i^2}{2}\right)\left(s_0^2+\frac{s_i^2}{2}\right)}{\frac{s_i^2}{2}+\frac{s_i^2}{2}+s_0^2+\frac{s_i^2}{2}} = 0.1095.$$

(It will be noted that av. K_s=av. K_0=15.46.)

TABLE II

A comparison between weighted, $[V_w']$, and non-weighted, $[V_s]$ and $[V_0]$, estimates

Occasion	Absorbances			K_s	K_0	$\frac{K_s^2}{K_0^2}$	Estimates (μg/g)		
	Unknown*	Standard					V_s	V_0	V_w'
I	0.210	0.263	0.235	16.06	15.42	1.08	3.37	3.24	3.32
II	0.262	0.237	0.217	17.62	15.31	1.32	4.62	4.01	4.35
III	0.283	0.300	0.276	13.89	15.56	0.80	2.93	4.40	4.08
IV	0.230	0.236	0.298	14.98	15.49	0.93	3.45	3.56	3.49
V	0.250	0.304	0.352	12.20	15.68	0.61	3.05	3.92	3.28
VI	0.205	0.251	0.261	15.63	15.45	1.02	3.20	3.16	3.18
VII	0.290	0.212	0.265	16.74	15.37	1.19	4.85	4.45	3.68
VIII	0.274	0.322	0.335	12.16	15.68	0.60	3.33	4.30	3.58
IX	0.205	0.307	0.229	14.93	15.49	0.93	3.06	3.18	3.11
X	0.263	0.298	0.292	13.56	15.59	0.76	3.57	4.10	3.74
XI	0.205	0.216	0.182	20.10	15.15	1.76	4.12	3.10	3.60
XII	0.272	0.245	0.207	17.70	15.31	1.33	4.81	3.16	4.52
XIII	0.243	0.216	0.262	16.74	15.37	1.19	4.07	3.73	3.93
XIV	0.242	0.247	0.282	15.09	15.48	0.95	3.65	3.75	3.69
XV	0.261	0.259	0.276	14.93	15.49	0.93	3.90	4.04	3.97
XVI	0.220	0.259	0.267	15.21	15.48	0.97	3.35	3.41	3.37

*Average of two values.

Then the standard deviation of V_s is 0.418, of V_0 is 0.542, and of V_w' is 0.331. The precision of the analysis was therefore improved by the derivation of the combined estimate.

A Terminological Note

The definition of the variability in colorimetric determinations may be facilitated with the introduction of a new term. An analytical procedure in which the variance of the simultaneous replicates (intravariance) is negligible (e.g., it is smaller than the variance which corresponds to the desired level of precision of the determination) may be called *antistoichic*. The term intends to convey the meaning that a quantitative correspondence between reagents and colored products of the reaction is obtained on simultaneous treatment of replicates. Antistoichia should not be considered synonymous either to reproducibility or to stoichiometry. An analytical colorimetric procedure is reproducible when the variability of the absorbances of replicates analyzed either on the same or on different occasions is negligible (that is, one sample is enough to give the desired precision in the analysis). A poorly reproducible procedure may be antistoichic if the variability of the absorbance of simultaneous replicates is negligible, whereas the variability of the absorbance of

replicates analyzed on different occasions is substantial. A poorly antistoichic procedure is, therefore, always poorly reproducible but a poorly reproducible procedure may or may not be antistoichic. The designation of a colorimetric procedure as stoichiometric (a term often used in colorimetry) is better to be reserved only for a procedure in which the ratio of the amounts of the yielded colored product(s) to the amounts of the reagents can be calculated directly by the molecular equations of the reactions on which the procedure is based.

Poorly reproducible and poorly antistoichic colorimetric procedures may be useful analytically as long as they give, on the average, increasing absorbances with increasing concentrations of the substance to be determined. We may call such procedures partially reproducible and partially antistoichic, giving a quantitative meaning to reproducibility and to antistoichia. Measurements of the deviations of a partially reproducible and partially antistoichic determination from complete reproducibility and antistoichia are given respectively by the total variance (intervariance plus intravariance) and intravariance of the absorbances.

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USE OF GLUTAMIC ACID-U-C^{14} TO DETERMINE NUTRITIONALLY ESSENTIAL AMINO ACIDS FOR LARVAE OF THE BLOW FLY, PHORMIA REGINA[1]

R. KASTING[2] AND A. J. MCGINNIS[3]

Abstract

The production of $C^{14}O_2$ by third-instar larvae of the blow fly, *Phormia regina* Meig., after it was injected with glutamic acid-U-C^{14}, indicates that this substrate was metabolized under these conditions. However, the nutritionally essential amino acids lysine, phenylalanine, valine, isoleucine, leucine, and threonine, isolated from the injected larvae, contained little radioactivity. A low level of radioactivity in arginine, histidine, and methionine suggests that they were slowly synthesized. The nutritionally non-essential amino acids alanine, serine, aspartic acid, and proline contained large quantities of radioactivity; tyrosine and glycine were exceptions. These results, in agreement with earlier work that used glucose-U-C^{14}, show that radioactivity data are useful for determining certain of the nutritionally essential amino acids.

Introduction

In 1952, Steele (1) showed that carbon-14 appeared in the nutritionally non-essential but not in the nutritionally essential amino acids after sucrose-U-C^{14} was metabolized by a mouse. This has been confirmed more recently with other organisms (2, 3). These observations provided the basis for an indirect method of determining which amino acids are essential in diets of organisms that cannot be reared on chemically defined media (4, 5).

The indispensable amino acids for larvae of the blow fly, *Phormia regina* Meig., have been determined by the classical deletion procedure (6, 7). Application of the indirect method to the blow fly using glucose-U-C^{14} as substrate gave results that generally agreed with those of the classical procedure (4). It appears that amino acids supplied in the diet of blow fly larvae provide carbon atoms for biosynthesis because the larvae thrive on liver and artificial diets devoid of carbohydrates (6, 8). Thus L-glutamic acid-U-C^{14} was used in the present study to determine whether the substrate that was provided influenced the labelling of the amino acids.

Materials and Methods

Blow flies (*P. regina*) were obtained from a culture that was collected originally in the Lethbridge area and maintained in the laboratory for numerous generations. The larvae that were used in the experiments were reared on fresh pork liver in a room maintained at 25° C.

About 60–72 hours prior to pupation the larvae were anaesthetized with CO_2. A No. 27G, ⅜-in. hypodermic needle attached to a 1.0-ml syringe in a ratchet-type microinjector* was used to inject the radioactive solution. The

[1]Manuscript received June 9, 1960.
Contribution from the Chemistry and the Entomology Sections, Canada Agriculture Research Station, Lethbridge, Alberta.
[2]Chemistry Section.
[3]Entomology Section.
*Purchased from Dr. W. C. Fest, P. O. Box 127, Masonville, New Jersey, U.S.A.

needle, directed toward the anterior end of the larva, was inserted into the posterior third of the insect body. About 6 μl of solution were injected into each larva, but in some instances fluid was lost from the puncture when the needle was removed. The radioactive solution was prepared by dissolving 0.65 mg (100 μc) of L-glutamic acid-U-C^{14} (Atomic Energy of Canada Limited) in 10 ml of distilled water.

Fifty larvae in each of two groups were injected with the glutamic acid-U-C^{14} solution. After injection the larvae were put in test tubes containing fresh pork liver and sawdust. The tubes were placed in a container equipped to collect respired CO_2. The collection train consisted of three absorption bottles, which contained, in order, 100 ml each of 0.5 *M* KOH, 1.0 *M* $BaCl_2$, and 0.5 *M* $BaCl_2$. After 3, 6, 12, 24, and 48 hours the solutions were replaced, sampled, and analyzed for radioactivity.

After injection the larvae in group I were maintained for 63 hours and supplied daily with fresh liver and sawdust; those from group II, progeny of a later generation of the laboratory culture, were kept on the same liver and sawdust for 48 hours. Twenty-eight living insects, ranging from late larval to early pupal stages, and 18 living insects in the chalky white prepupal stage were recovered from groups I and II, respectively. After the postinjection period of metabolism the insects were rinsed several times with distilled water, quick-frozen, and stored at −30° C.

Extraction and Isolation of Amino Acids

The frozen larvae were dropped into 25 ml of boiling 80% ethanol and ground in a Potter–Elvehjem homogenizer for 10 minutes. After centrifugation, the residue was subjected to a second ethanol extraction, followed by one with 25 ml of boiling water; the three extracts were combined and reduced to dryness *in vacuo* at 40°–50° C in a rotary evaporator. Water was added and after centrifugation an aqueous extract and a small amount of solid residue were separated. This residue was combined with the original residue and the whole was hydrolyzed with 6 *M* HCl under reflux for 40 hours. The acid was removed *in vacuo* at 40°–50° C and the soluble components were dissolved in a small volume of water and combined with the extract previously obtained.

The total volume of extract from group I insects, about 75 ml, was placed on a Dowex-50-H^+ ion-exchange column (2.5 cm×50 cm). The column was eluted with 1 liter of CO_2-free water. Then five hundred 2.5-ml fractions were collected by gradient elution with hydrochloric acid (9). Every fifth tube was evaporated to dryness by a stream of hot air; 0.2 ml water was added and 1/10th of the solution was chromatographed in one dimension on Whatman No. 1 paper with *n*-butanol/acetic acid/water (4:1:5). Appropriate known amino acids were also chromatographed on the same sheets. These chromatograms in conjunction with published elution curves were used as a guide for bulking fractions that contained a particular amino acid. The bulked fractions were evaporated to dryness *in vacuo*, at 40°–50° C, redissolved in a small amount of water, and band chromatographed.

The amino acids from the combined extracts of group II insects were

separated by band chromatography without prior separation on an ion-exchange column.

Combined extracts, prepared and bulked as described above, were obtained on a group of non-injected larvae at a similar stage of development to those in group II and were used to obtain the quantitative data presented in Table II.

Bulked fractions were band chromatographed in the long direction on 46×57 cm Whatman No. 1 paper that had been previously washed with 1% oxalic acid and water. Fractions containing an amino acid of interest were chromatographed successively in *n*-butanol/acetic acid/water (4:1:5), 95% ethanol/concentrated ammonium hydroxide (95:5), water-saturated phenol, and 95% ethanol. When particular amino acids were not adequately separated in these four solvents, others were used between the phenol and ethanol runs as indicated below:

Amino acids separated	Solvent
Glycine, serine, aspartic acid	Methyl ethyl ketone/concentrated ammonium hydroxide/2-butanol/water (3:1:5:1)
Glutamic acid, aspartic acid*	2-Butanol/88% formic acid/water (120:1:40)
Valine, methionine	95% ethanol/acetic acid (95:5)
Leucine, isoleucine	Methyl ethyl ketone/concentrated ammonium hydroxide/2-butanol/water (3:1:5:1)

After development of a chromatogram the band containing a particular amino acid, as located by marker strips, was removed and eluted with water. The eluate, after concentration *in vacuo* at 40°–50° C, was reapplied as a band to a fresh sheet of paper for development in the next solvent. Papers without added amino acids were treated in a similar manner to obtain bands that served as controls for analyses.

After the amino acids were eluted from the paper bands with water, the solutions were evaporated to dryness and the residue dissolved in a known volume of water. Radioactivity determinations on aliquots of these solutions were obtained as described later. To permit calculations of specific activities the quantities of amino acids were determined on aliquots by using specific or semispecific methods for proline (10), tyrosine (11), arginine (12), methionine (13), histidine (11), phenylalanine (14), and a general ninhydrin method (15) for the others.

The quantitative results for aspartic acid, glutamic acid, serine, glycine, valine, threonine, and alanine shown in Table II were obtained by a two-dimensional chromatographic method (16). Quantitative determinations of the other amino acids were conducted on the combined extracts by the specific and semispecific methods described above except for phenylalanine and tyrosine, which were first partially purified by band chromatography in *n*-butanol/acetic acid/water (4:1:5).

Radioactive Counting Procedures

The quantity of radioactivity in the CO_2 was determined on aliquots of the combined $BaCl_2$–KOH trap solutions dried under an infrared lamp in stainless-steel cupped planchets. Corrections for self-absorption were made as described by Calvin *et al.* (17).

*Three passes of the solvent.

Except where noted, the radioactivities of amino acids were determined on aliquots containing at least 1 μmole of the compound applied to copper planchets and dried under an infrared lamp. Because the weights of the amino acids were negligible, no corrections were made for self-absorption. The activities per micromole of carbon were calculated and results are reported on this basis.

All radioactivity measurements were made in a windowless gas-flow counter (Tracerlab Incorporated) using a 99% helium – 1% isobutane mixture of gas.

Results and Discussion

Injected glutamic acid-U-C^{14} was readily metabolized by both groups of larvae as indicated by the evolution of appreciable quantities of $C^{14}O_2$, most of which appeared within 24 hours after injection. For example, with group II larvae the percentages of $C^{14}O_2$ evolved at different time intervals during the 48 hours after injection were as follows: 3 hours, 45%; 6 hours, 55%; 12 hours, 80%; 24 hours, 95%.

Of the radioactivity injected into the group II larvae, about 50% was recovered as $C^{14}O_2$, 15% in the combined extracts, and less than 1% in the humin after hydrolysis. About 30% of the injected radioactivity was present in the liver and sawdust after the insects were removed. Presumably this was due to excreted and exuded radioactive substances.

The results in Table I indicate that this organism can synthesize certain amino acids more readily than others. Comparison of specific activity data with the reported requirements (Table I) showed that six essential amino

TABLE I

Specific activities of amino acids isolated from larvae of the blow fly, *P. regina*, after injection with glutamic acid-U-C^{14}

Amino acid	Reported requirement (ref. 6, 7)	Specific activities (c.p.m./μmole C)	
		Group I	Group II
Proline	+	19.7	49.1
Alanine	−	11.9	10.0
Aspartic acid	−	5.6	26.0
Serine	−	3.4	2.9
Glycine	−	0.9	3.9
Arginine	+	0.7	0.7
Histidine	+	0.5	<1
Methionine	+	—	0.2
Lysine	+	0.06	—
Phenylalanine	+	0.09	0.13
Valine	+	0.07	0.08
Isoleucine	+	0.08	—
Leucine	+	0.06	—
Threonine	+	*	—

*Not different from background.

acids, lysine, phenylalanine, valine, isoleucine, leucine, and threonine, contained negligible quantities of radioactivity. By comparison three of the nonessential amino acids isolated from the larvae, alanine, aspartic acid, and

serine, were highly labelled. There was at least a 20-fold difference in the levels of radioactivity between the essential and non-essential amino acids mentioned above. These findings generally agree with results of earlier work (4) on the blow fly in which glucose-U-C^{14} was injected. In addition, glutamic acid, the metabolite used in the present study, was labelled.

Table I shows that proline isolated from larvae injected with glutamic acid-U-C^{14} had the highest specific activity of the amino acids investigated. Earlier work (4) had shown that labelled proline was synthesized when glucose-U-C^{14} was injected into larvae of the blow fly. Classical nutritional studies with this organism (6) had indicated that proline was a dietary requirement, a result inconsistent with the labelling data. This discrepancy prompted an investigation of the proline requirement of the local strain of blow fly using the classical deletion procedure. Results (unpublished) clearly showed that the local strain of *P. regina* did not require proline in the diet.

The levels of radioactivity in arginine, histidine, and methionine, as shown in Table I, are intermediate between those of the essential and non-essential amino acids discussed above. Arginine, histidine (6), and either methionine or cystine (7), are nutritionally essential for larvae of the blow fly. Consequently, even lower levels of radioactivity were expected in these amino acids. The quantities of radioactivity found in arginine, histidine, and methionine suggest that some synthesis does occur but presumably the rate is too slow to support growth.

The specific activities of cystine and tyrosine were not measured with the same degree of accuracy as the amino acids listed in Table I. At most 1 c.p.m. per μmole C was present, a level sufficiently low to classify these amino acids as essential. This classification agrees with nutritional studies for cystine (7) but not for tyrosine (6). If, however, the blow fly synthesizes tyrosine primarily from phenylalanine, as does the silkworm (18), then the low count would be expected.

The glycine isolated from group II insects contained sufficiently large quantities of radioactivity to classify it as nutritionally non-essential. This agrees with the conclusions drawn from classical nutritional studies (6) and the indirect method when glucose-U-C^{14} was used (4). However, glycine from group I insects had a specific activity similar to that of arginine, a result that suggests that glycine is nutritionally essential. The reason for the relatively different levels of radioactivity in glycine isolated from the two groups of insects is not known. The stage of larval development when injected and (or) the time interval between injection and subsequent processing of the larvae may have affected the level of the specific activity. It is of interest that, although three of the amino acids from group II insects, glycine, proline, and aspartic acid, had higher specific activities than those isolated from group I, alanine and serine were not different. Before these observations can be interpreted it will be necessary to determine how the specific activity of the different amino acids varies with time after injection of the labelled substrate.

Table II shows that the larvae at a stage of development comparable with

TABLE II

Relative quantities of some amino acids present in prepupae of the blow fly, *P. regina*

Amino acid	Concentration (μmole/g)*	Amino acid	Concentration (μmole/g)*
Aspartic acid	440	Methionine	80
Serine	260	Tyrosine	60
Glycine	160	Threonine	50
Glutamic acid	130	Alanine	50
Valine	130	Histidine	50
Proline	80	Phenylalanine	40
Arginine	80		

*Gram fresh weight of prepupae.

those of group II contained aspartic acid, serine, glycine, glutamic acid, and valine in the greatest concentrations. Thus dilution may partly account for the lower specific activities of some of the amino acids, such as valine and glycine, but it cannot account for all the low specific activities obtained. If the values in Table II are representative of the quantities present in the insects of group I and group II, it may be calculated that about 97 and 99%, respectively, of the radioactivity in the isolated amino acids occurred in aspartic acid, proline, serine, glycine, and alanine, all of which are nutritionally non-essential.

Conclusions

The use of glutamic acid-U-C^{14} as a substrate to determine nutritionally essential amino acids for larvae of the blow fly, *P. regina*, led to the same conclusion as when glucose-U-C^{14} was used (4): the levels of radioactivity in isolated amino acids were relatively high in those which were nutritionally non-essential and low in those required in the diet. Certain results indicate that care must be exercised in their interpretation. Thus it is possible by this method to determine the nutritionally essential amino acids for organisms that cannot yet be reared on chemically defined diets.

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PREPARATION AND EVALUATION OF POLYSTYRENE–ANTIGEN CONJUGATES FOR THE ISOLATION OF ANTIBODIES[1]

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Abstract

Antigenically specific adsorbents were prepared by coupling soluble protein antigens to a polystyrene-supporting medium by azo bonds. These immunosorbents were shown to combine specifically with homologous precipitating rabbit antibodies. Elution of precipitating antibodies from the immunosorbents was achieved by dissociation with hydrochloric acid at pH 3. Under optimal conditions 35% of the antibodies removed from the antisera were recovered with a purity of over 80%. Electrophoretic analyses showed that precipitating rabbit antibodies were localized primarily in the region of gamma-globulins. The heterogeneity of the antigen–antibody systems studied was demonstrated by a combination of immunochemical, physicochemical, and radioactive tracer methods.

Introduction

In the large number of studies devoted to the isolation of antibodies in a pure form, different supporting materials have been used for the preparation of antigenically specific adsorbents. Landsteiner and van der Sheer (1) coupled diazotized haptens to red blood cell stroma. With these conjugates antibodies were removed from the corresponding antihapten sera and could be eluted with dilute acetic acid in fairly good purity. Campbell *et al.* (2) developed a more general method for the purification of antibodies by coupling protein antigens to a cellulose derivative. For the synthesis of this adsorbent, cellulose was reacted with *p*-nitrobenzyl chloride, the nitro groups of the polynitrobenzyl cellulose were reduced, and the resulting polyamino compound was diazotized. The polydiazonium derivative of cellulose, on reaction with protein antigens, formed insoluble cellulose–antigen conjugates. These preparations were shown to be useful for the isolation of antibodies in good yields (of the order of 90%) and in high purity (of about 90%). The same supporting material was also used by Talmage *et al.* (3) with good results for the purification of antibodies. However, other workers (4, 5) have not found this method too satisfactory, since, with similar cellulose–antigen adsorbents, the yield of antibody recovery was as low as 20 to 25% and the purity of the recovered antibody did not exceed 50% (4).

Ion-exchange resins have also been used as starting materials for the preparation of insoluble immunologic adsorbents. Isliker (6) converted carboxylated or sulphonated resins to the corresponding acyl or sulphonic chlorides which were then reacted with protein antigens. The resulting conjugates were shown to remove antibodies from the corresponding antisera but the recovery of antibodies varied according to the batch of the conjugate used.

Karush and Marks (7) coupled diazotized haptens to fibrinogen, a protein

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Contribution from the Departments of Biochemistry and Chemistry, McGill University, Montreal, Que. The preliminary results of this work were originally presented at the Annual Conference of the Chemical Institute of Canada, Vancouver, 1957. Submitted by L. G. to the Department of Biochemistry in partial fulfillment of the requirements for the Ph.D. degree.

which can be salted out much more readily than the antibody. Incubation of this fibrinogen–hapten conjugate with the antiserum resulted in the formation of a precipitate from which antihapten antibodies could be dissociated by a solution of the free hapten. After precipitation of fibrinogen with ammonium sulphate, 50% of the antibodies present in the original serum was recovered, 90% of which was specifically precipitable with the antigen. This procedure can be considered as a modification of the method developed by Campbell *et al.* (8) in which a polyhaptenic dye was used as antigen to form a specific precipitate with the homologous antibody. In the latter method the simple hapten was used to displace the polyhaptenic molecule from its complex with antibody; the polyhaptenic dye was precipitated at pH 3.2 and pure antibody was recovered from the supernatant solution after removal of the hapten by dialysis.

The supporting media used in these studies possessed ion-exchange properties and, therefore, it is conceivable that serum proteins may have become bound to them non-specifically. In order to avoid, or at least to minimize, non-specific adsorption of serum proteins, in the present study protein antigens were coupled through stable azo bonds to a non-polar polystyrene framework.*

Methods and Materials

Preparation of Polystyrene–Antigen Conjugates

For the synthesis of immunosorbents four polystyrene preparations† of different molecular weight distributions were used as starting material: PS-2 (M.W. 30,000), PS-3 (M.W. 55,000), Q-752-8 (M.W. 140,000), and Styron 665 (M.W. 210,000). The reactions involved in the preparation of polystyrene–antigen conjugates are shown in the flowsheet presented in Fig. 1.

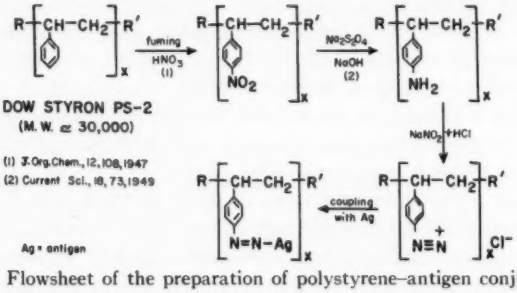

FIG. 1. Flowsheet of the preparation of polystyrene–antigen conjugates.

The polystyrene beads were first dissolved in N,N-dimethylformamide, the solution was then poured into water, and the precipitated polystyrene was dried and pulverized. This finely divided polystyrene powder was nitrated according to the method of Bachman *et al.* (10): 5 g of polystyrene were added slowly to 50 ml of fuming nitric acid and the mixture was stirred. During

*The preliminary results of this work were published earlier (9).

†Obtained through the courtesy of the Physical Laboratory, Dow Chemical Co., Midland, Michigan.

nitration the polystyrene dissolved with evolution of heat. To keep the temperature below 50° C, the nitration was performed in a water bath at about 25° C. The reaction was stopped (after 15 to 60 minutes) by pouring the solution into cold water. This resulted in the precipitation of the light yellow colored polynitro polystyrene.

Reduction of the polynitro derivative of polystyrene with zinc and hydrochloric acid or by direct hydrogenation, catalyzed by finely divided nickel,* was attempted. The reduction of polynitro polystyrene was performed in absolute alcohol at room temperature and at a hydrogen pressure of 10 lb/sq. in. for 5 hours, using about 3 g of catalyst for 15 g of polynitro polystyrene. These methods, however, did not lead to a substantial reduction of the nitro groups. Furthermore, these procedures proved unsatisfactory since the metal ions (of zinc or nickel) appeared to complex with the polyamino polystyrene. These procedures were, therefore, abandoned and the reduction was performed either with sodium hydrosulphite (10, 12) or with thiourea dioxide (13). In the former method 5 g of polynitro polystyrene was suspended in 200 ml of 2 *N* ammonium hydroxide or sodium hydroxide, 12 g of sodium hydrosulphite was added, and the mixture was heated for 3 days in a boiling water bath. In the other method the reduction was performed in 200 ml of 10% sodium carbonate solution with 8 g of thiourea dioxide at 60° C for 3 to 4 hours. The polyamino derivative of the polystyrene of lower molecular weight was soluble in hydrochloric acid whereas the polyamino polystyrenes of higher molecular weight were insoluble.

The number of amino groups in the polyamino polystyrene was determined by titration with 1 *N* HCl. For diazotization, a slight excess of sodium nitrite and 3 equivalents of hydrochloric acid were used and the reaction was carried out at 0° C. The completion of the reaction was established in the conventional manner with starch-iodide paper. The pH of the solution containing the polydiazonium salt was then adjusted to 7.5 with sodium bicarbonate and the protein antigen to be coupled was added slowly with stirring. In general, 0.1 to 0.5 g of antigen was used per gram of polyamino polystyrene. The polystyrene–antigen conjugate was obtained as a dark brown or yellow brown precipitate.

Free unreacted diazo groups could be detected by reaction with β-naphthol; if present, they were destroyed by resuspending the polystyrene–antigen conjugate in a fresh antigen solution or simply in 0.9% saline for a period of 10 to 14 days. On several occasions the diazonium groups were blocked by reaction with glycine, which did not change perceptibly the color of the polystyrene–antigen conjugate. The completeness of the latter reaction was demonstrated by the absence of a color change on testing with β-naphthol.

Isolation of Antibody

For the isolation of antibody the polystyrene–antigen conjugates were suspended in the appropriate antiserum for 1 to 3 hours with occasional stirring. In general, conjugates prepared with 0.5 g of polyamino polystyrene were

*The catalyst was prepared from Raney nickel according to the classical method (11).

capable of removing antibodies from 2 to 5 ml of antiserum. The suspension was then put in perforated plastic tubes provided with filter pads. Each plastic tube was placed in a 40-ml glass centrifuge tube on three glass prongs protruding from the wall of the tube about 2.5 cm from the bottom of the tube. The suspension was centrifuged at 2000 r.p.m. for 5 minutes. The filtrate was then tested for the presence of antibody. The polystyrene–antigen–antibody complex was left in the plastic tube, washed with saline, and recentrifuged. This procedure was repeated until the washing was found to be free of protein. The polystyrene–antigen–antibody complex was then transferred to a small beaker and was suspended in saline. The pH was adjusted to 3 with dilute hydrochloric acid and the suspension was incubated for 90 to 120 minutes at 4° C or at room temperature. The dissociated antibody was separated from the polystyrene–antigen conjugate by centrifugation as described above. The pH of the solution, referred to hereafter as eluate, was adjusted to 7. Occasionally, at this stage a small flocculent precipitate was formed. This artifact, however, could be eliminated by treating the polystyrene–antigen conjugate with hydrochloric acid prior to its suspension in the appropriate antiserum. The polystyrene–antigen conjugates, after completion of an experiment, were resuspended in saline and stored at 4° C for other experiments.

Determination of Antibody Content

The antibody content of the whole antisera and of the eluates was determined by quantitative precipitin tests performed according to the method of Heidelberger and Kendall (14). One-milliliter aliquots of antiserum (or eluate) were added to a series of tubes each containing decreasing amounts of the homologous antigen in 1-ml volume. The antiserum was used in a dilution to give a maximum precipitate of about 100 μg/ml, as determined from a preliminary experiment. After mixing of the two reactants, the solutions were incubated at 37° C for 2 hours, followed by incubation at 4° C for 48 hours. The precipitates were then collected by centrifugation and washed three times with 2-ml aliquots of chilled saline. The washed precipitates were digested with sulphuric acid and were analyzed for nitrogen either by the Nessler colorimetric method according to the procedure of Lanni *et al.* (15) or by the micro-Kjeldahl method (16). The precipitin curves were obtained by plotting the amount of specific precipitate against the amount of antigen added.

Free Electrophoresis

Electrophoretic analyses were carried out in the Spinco Model H Tiselius apparatus at 0.8° C. Prior to electrophoresis, all serum samples were dialyzed against veronal buffer of pH 8.6, ionic strength 0.1, for 24 hours and were diluted with the same buffer to a protein concentration of about 1 gram%. The total protein and the relative concentrations of the various components were calculated from photographs of the Schlieren patterns and of the Rayleigh interference fringes of the ascending boundaries taken at the end of the experiments (17).

Antigen–Antibody Systems Used

Antisera were produced in rabbits against different protein antigens, such as

normal human serum (NHS), human serum albumin (HSA), human serum gamma-globulins (HGG), bovine serum albumin (BSA), and a water-soluble extract of ragweed pollen (WSR). Albino rabbits of 3 to 4 kg were injected intravenously (marginal ear vein) with 1 ml of a 1% solution of the antigen three times per week for 4 weeks. Blood samples were collected periodically starting 6 days after the last injection and were tested by ring test for antibody content. When a good titer had been reached, 50 to 70 ml of blood were taken from each rabbit from the marginal ear vein. The blood samples were allowed to clot at room temperature and were then placed in the cold to allow the clot to retract. After clotting, the bloods were centrifuged, and the sera were decanted and sterilized by filtration through Swinney-type Seitz filters and stored at 4° C until used.

In view of the poor antigenicity of ragweed, the pollen extract was precipitated on aluminum hydroxide by the method of Proom (18). Ten milliliters of a sterile solution of the antigen (containing 1 g of WSR) was added to 90 ml of a 10% potassium alum solution. The pH was adjusted to 6.5 by the addition of 5 *N* sodium hydroxide. The alum and sodium hydroxide solutions were sterilized prior to mixing. The resulting suspension was centrifuged and the supernatant discarded. The precipitate was washed twice with 180 ml of sterile saline and was finally resuspended in 180 ml of sterile saline containing merthiolate in a concentration of 1:5000. This suspension was injected intravenously into rabbits used for immunization according to the schedule described above.

Results

Although the nitration of polystyrene could be performed with ease, as stated previously the reduction of the polynitro polystyrene was associated with some difficulties. Attempts to reduce the nitro derivative with zinc and hydrochloric acid or by catalytic hydrogenation in the presence of Raney nickel met with little success. Better results were obtained when sodium hydrosulphite was used for reduction. With the polystyrene of a molecular weight distribution of 30,000 (PS-2), the polyamino polystyrene was obtained as a brown crystalline powder, soluble in dilute hydrochloric acid. From the titration of this compound with 1 *N* HCl it was found that on the average there were two amino groups per five styrene residues. This result was confirmed by nitrogen values obtained by digesting the polyamino polystyrene with sulphuric acid and determining the nitrogen content as ammonium sulphate.

It was observed, however, that under the conditions required for reduction (i.e. strongly alkaline medium, prolonged heating at 100° C), the formation of polyamino polystyrene was occasionally accompanied by undesirable side reactions. Thus, in some instances the resulting polyamino polystyrene was soluble in dilute alkali, which could have been due to the production of carboxyl groups by the oxidation of polystyrene.

In order to reduce the polynitro polystyrene under milder conditions, thiourea dioxide was used as reducing agent in a less alkaline medium (10%

sodium carbonate solution) and the reaction was carried out at lower temperature (60° C). However, with this method only partial reduction of the polynitro polystyrene could be achieved. This conclusion was arrived at from the differences observed between the nitrogen content of the polyamino polystyrene preparations and the number of amino groups determined from titration. With minor changes in the experimental conditions, such as variation of the time of nitration and of the amount of reducing agent, polyamino polystyrene derivatives could be prepared having one amino group per 6 to 15 styrene residues.

Several polystyrene–antigen conjugates, prepared with the various batches of polyamino polystyrene and with different antigens such as HSA, HGG, NHS, BSA, and WSR, removed completely precipitating antibodies from the homologous rabbit antisera. The specificity of this reaction was demonstrated by the fact that these antisera were not depleted of their antibodies by conjugates prepared with heterologous antigens. For example, incubation of a polystyrene–NHS conjugate with a rabbit anti-WSR serum had no effect on the antibody content of the antiserum. Electrophoretic analyses of this serum before and after exposure to the polystyrene–NHS conjugate demonstrated that no significant change occurred in the composition of the serum following treatment with a heterologous polystyrene–antigen conjugate (see Fig. 2). Furthermore, it can also be seen that the amount of protein bound nonspecifically by the immunosorbent was negligible (of the order of 0.01 gram%).

On the other hand, when the polystyrene–NHS conjugate was incubated with the homologous rabbit antiserum, i.e. with anti-NHS serum, all anti-

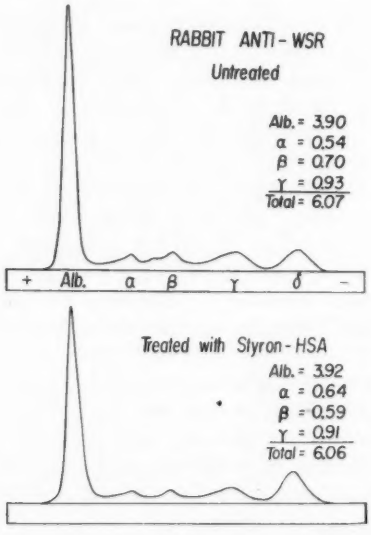

FIG. 2. Electrophoretic analysis of a rabbit antiserum before and after exposure to a heterologous polystyrene–antigen conjugate. WSR and HSA designate water-soluble constituents of ragweed pollen and human serum albumin, respectively.

bodies were removed specifically. From free electrophoretic analyses of the whole serum and of the serum treated with the homologous polystyrene–antigen conjugate (Fig. 3), it follows that about 13 mg of protein per ml were

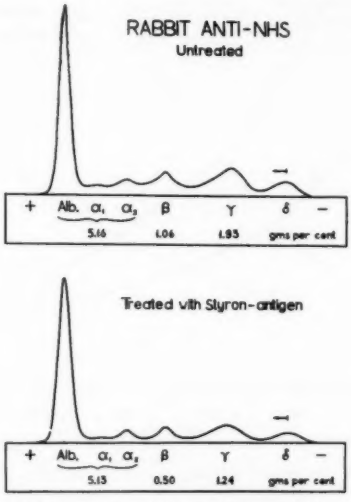

FIG. 3. Electrophoretic analysis of a rabbit antiserum before and after exposure to the homologous polystyrene–antigen conjugate. NHS represents normal human serum.

removed from this antiserum. Furthermore, it appears that the antibodies removed by the immunosorbent were localized primarily in the region of gamma-globulins and extended also into the region of the faster migrating serum globulins.

Recovery of precipitating antibodies from polystyrene–antigen–antibody complexes was achieved by dissociation with hydrochloric acid at pH 3. As mentioned earlier, the removal of antibodies from the antisera could be effected with any of the homologous polystyrene–antigen conjugates prepared with the different preparations of polyamino polystyrene. However, the extent of recovery of antibodies by dissociation from the polystyrene–antigen conjugates seemed to depend on the batch of polyamino polystyrene used for the preparation of the immunosorbent. Thus, using a highly aminated polystyrene derivative (having one amino group per two styrene residues, prepared from a polystyrene batch with an average molecular weight of 30,000), the behavior of the eluted antibodies differed from that present in the whole original antiserum. The precipitin curves of this system (BSA–anti-BSA) are illustrated in Fig. 4. As can be seen, for maximum precipitation of the eluted antibody the concentration of the antigen was about 40 times higher than that required for the precipitation of antibodies from the whole antiserum. Essentially the same results were obtained with another antigen–antibody system (i.e. HGG–anti-HGG) when a polystyrene–antigen conjugate, prepared with an aliquot of the same batch of polyamino polystyrene, was used (see Fig. 5). The precipitin

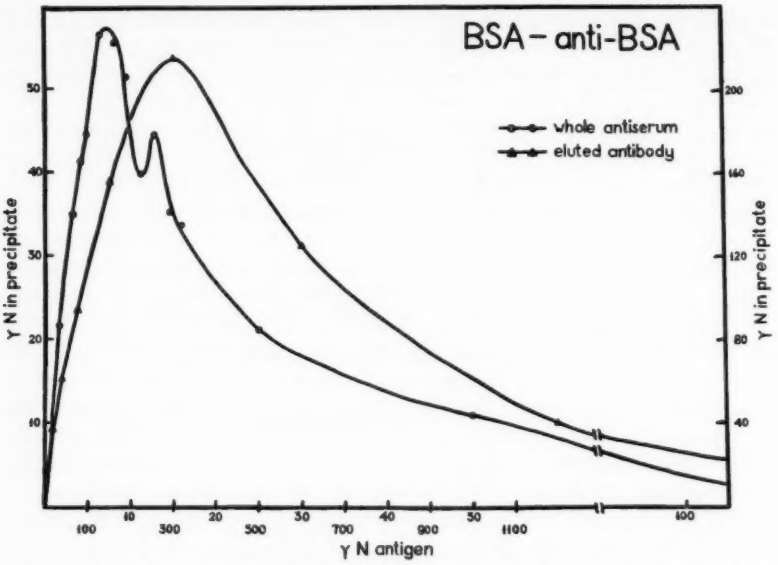

FIG. 4. Precipitin curves obtained with whole antiserum and with antibodies eluted from the immunosorbent. The upper scale of the abscissa and the left ordinates refer to the curve obtained with the whole antiserum; the lower scale of the abscissa and the right ordinates refer to the curve obtained with the eluted antibody. BSA designates bovine serum albumin.

curve given by the eluted antibody preparation showed the same characteristics as the corresponding curve in the previous system, i.e. maximum precipitation occurred at an antigen concentration 40 times that required at the optimum zone for the whole antiserum and the total amount of precipitate formed with the eluted antibody was higher than that given by the whole antiserum. It can also be seen in Fig. 5 that treatment of the whole antiserum with hydrochloric acid (at pH 2 or 3) did not shift the optimum zone towards higher concentrations of the antigen. However, depending on the pH used and the time of treatment, the amount of precipitate varied. Thus, treatment of the antiserum at pH 2 for 3 hours resulted in an increase in precipitate of about 30% (see Fig. 5). On the other hand, under the conditions used for the elution of antibodies from the polystyrene–antigen–antibody complexes, i.e. at pH 3 for 90 minutes, the precipitin curve was identical with that obtained with the untreated antiserum.

In view of the unusual behavior of these eluted antibodies, the yield and purity of the preparation could not be estimated from the precipitin curves. In an attempt to determine the amount of antibody removed from the antiserum by the immunosorbent, electrophoretic analyses were performed on the untreated antiserum and on the serum treated with the homologous polystyrene–antigen conjugate. The results of this experiment are shown in Fig. 6. As can be seen, about 36 mg protein/ml, consisting of 1.8 mg of albumin, 5 mg

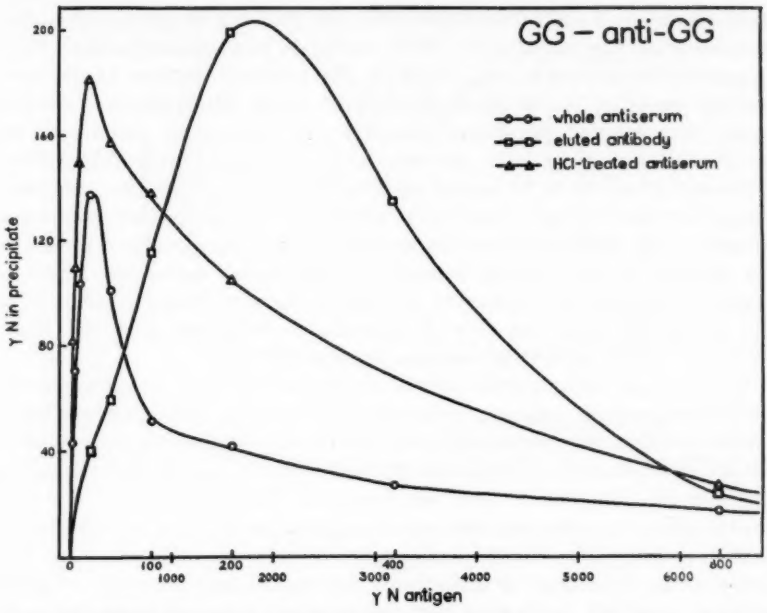

FIG. 5. Precipitin curves obtained with whole antiserum and with antibodies eluted from the immunosorbent. The upper scale of the abscissa refers to the curves obtained with the whole antiserum and with the antiserum treated with hydrochloric acid at pH 2 for 3 hours; the lower scale of the abscissa refers to the amounts of antigen used for the precipitation of eluted antibody. GG represents human serum gamma-globulins.

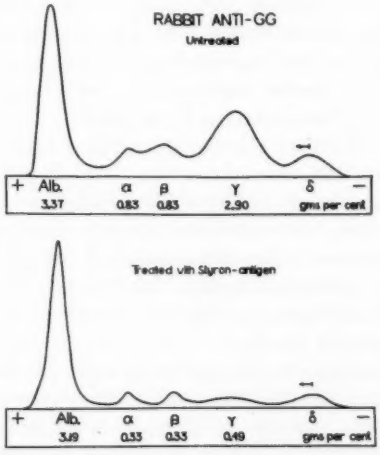

FIG. 6. Electrophoretic analysis of a rabbit antiserum before and after exposure to the homologous polystyrene–antigen conjugate. GG represents human serum gamma-globulins.

of alpha-globulins, 5 mg of beta-globulins, and 24.1 mg of gamma-globulins, were removed by the polystyrene–HGG conjugate from the antiserum. From the quantitative precipitin test, however, the antibody content of the antiserum was found to be 14 mg antibody/ml.* Since electrophoretic analysis indicated that most of the serum globulins and a negligible amount of the serum albumin were removed by the immunosorbent, it is questionable whether the globulins in excess of 14 mg/ml were removed by non-specific adsorption on the polystyrene–antigen particles or whether they represent antibodies not measured by the standard precipitin method. The disagreement of these two values, as well as the unusual behavior of the eluted antibodies, could be explained in terms of heterogeneity of these antigen–antibody systems. The results of the following two sets of experiments performed with the BSA–anti-BSA and HGG–anti-HGG systems support this view:

(i) Ten milliliters of anti-BSA serum were separated by zone electrophoresis on a starch-supporting medium, according to the method of Kunkel and Slater (19) and as described previously (20). When the separation between the fastest and slowest serum components reached about 35 cm, the electrophoresis was terminated. The starch block was then cut transversely into 1 cm wide segments and the protein was eluted from each segment. The distribution of antibodies amongst the various serum subfractions was determined by precipitation with the antigen in a semisolid medium of agar–agar (21). It was found that anti-BSA antibodies were heterogeneous electrophoretically and were associated with two distinct subfractions of the gamma-globulins, i.e. with gamma-1-globulins and with the slowest gamma-2-globulins, the two subfractions being separated from each other by 8 cm.

(ii) HGG was labelled with I^{131} by the method of Gilmore *et al.* (22) and, on the average, two iodine atoms were introduced per gamma-globulin molecule. This labelled antigen was used in precipitin tests performed with the whole antiserum and with the eluted antibodies. The radioactivity of each of the washed precipitates and of the supernatants was determined in a well-type scintillation counter. The results obtained with the whole antiserum indicated that at the equivalence point, where all the antigen is expected to be in the precipitate, about 25% of the antigen was present in the supernatant. Furthermore, in another precipitin test where the antigen concentration was not varied by serial halving dilution but by small equal increments as indicated in Fig. 7, the precipitin curve was composed of three distinct peaks, as given by both the nitrogen content and the radioactivity of the precipitates.

The results of these experiments may be interpreted as indicating (i) a spectrum of antibodies differing in their physicochemical properties and (ii) heterogeneity of the antigen itself.

As mentioned earlier, the immunosorbents used for the experiments discussed above were prepared with a polyamino polystyrene preparation having two amino groups per five styrene residues. In order to investigate the effect of the number of amino groups of the supporting medium on the character-

*This was an unusually high antibody titer; in general, antisera prepared in this laboratory contained less than 6 mg of antibody per ml.

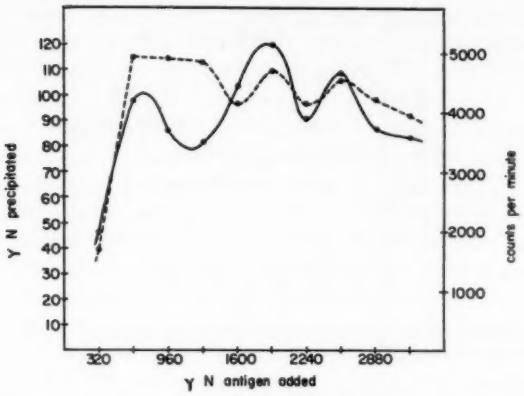

FIG. 7. Nitrogen content and radioactivity of specific precipitates consisting of I^{131} labelled HGG and antibodies eluted from the immunosorbent. The solid line represents the nitrogen content, the broken line represents the radioactivity of the specific precipitates.

istics of the immunosorbent, polyamino polystyrene derivatives with lower and varying degree of amination were synthesized. This was achieved by nitrating polystyrene for various periods (15, 30, 45 and 60 minutes) and by reducing the resulting nitro compounds by the different methods described previously. In this manner different batches of polyamino polystyrene preparations were obtained having one amino group per 6 to 15 styrene residues. Decrease in the number of the amino groups of the highly aminated polyamino polystyrene was also performed by partial diazotization and boiling in alcohol. This treatment results in the replacement of amino groups by hydrogen atoms.

None of these latter preparations (12 different polystyrene–antigen conjugates) gave satisfactory results. In some instances not only antibodies but also other serum proteins were adsorbed from the antisera, as determined by electrophoretic analyses. These artifacts led to the recovery of antibody preparations of low purity. In other cases only antibodies appeared to have been removed from the corresponding antisera but could not be recovered from the immunosorbents at pH 3.

Immunosorbents prepared with a commercial preparation of polyamino polystyrene of undisclosed physical and chemical properties* removed antibodies specifically from the homologous antisera, and the antibodies could be easily eluted from the polystyrene–antigen–antibody complexes by lowering the pH to 3. The precipitin curves obtained in an experiment with the whole antiserum (to HSA) and with the eluted antibody fraction are shown in Fig. 8. In this case the antibody content of the whole antiserum was 4.1 mg/ml. In contrast, the amount of precipitable antibody in the eluate was only 1.4 mg/ml, which amounts to a recovery of about 35%. However, it was gratifying to find that the total protein of the eluate was 1.7 mg/ml, which means that the purity of the eluted antibody preparation was about 82%.

*Obtained through the courtesy of Norsk Hydroelectric Co., Oslo, Norway.

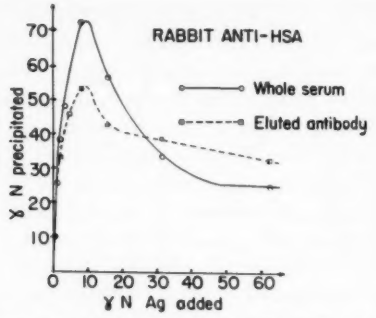

FIG. 8. Precipitin curves obtained with whole antiserum and with antibodies eluted from the immunosorbent. HSA designates human serum albumin.

Discussion

An ideal antigenically specific adsorbent should have a sufficiently high capacity for the antibody, should be insoluble under the experimental conditions used, and should not adsorb serum proteins non-specifically. These requirements were fulfilled by certain preparations of polystyrene–antigen conjugates which showed an unimpaired antigenic capacity with respect to the homologous antibodies and had very little or no affinity for non-antibody proteins. Whilst this work was in progress, similar findings were reported by other workers (23, 24) who used polystyrene–antigen conjugates. Grubhofer and Schleith (25) had also shown that enzymes could be coupled to diazotized polystyrene without impairing their enzymatic activity. More recently Kent and Slade (26) demonstrated that polystyrene–antibody conjugates could be synthesized and that the resulting conjugates had the ability of combining specifically with the corresponding antigens. Thus, polyamino polystyrene appears to possess a wide range of applicability in studies necessitating the fixation of a biologically active material by coupling the latter to an insoluble polymeric framework.

As mentioned previously, certain polystyrene–antigen conjugates displayed a high degree of non-specific binding of serum proteins. From the results of the chemical analyses (total nitrogen, number of diazotizable amino groups) of the batches of polyamino polystyrene used as starting material for the synthesis of these immunosorbents it would appear that non-specific binding of proteins occurred more frequently with adsorbents prepared with polyamino polystyrenes having a small number of amino groups. It would seem that the behavior of the polystyrene–antigen conjugates does depend on the degree of amination of polystyrene since this latter would determine the steric configuration of the three-dimensional polystyrene–antigen network. It is conceivable that in an aqueous medium the polystyrene–antigen conjugates prepared with batches of highly aminated polystyrenes retain an unfolded structure and that antibodies are combined only onto their surfaces. This type of conjugates should not retain proteins non-specifically. On the other hand, polystyrene molecules to which only a small number of antigen molecules have been at-

tached may be coiled into "spongy" structures, stabilized through the interaction of the hydrophobic benzene residues, which would not allow for the free draining of protein solutions through their matrices and thus lead to a nonspecific irreversible occlusion of proteins.

In the present study, elution of antibodies from polystyrene–antigen–antibody complexes was attempted by lowering the pH to 3. By this method, however, antibodies removed specifically from the antisera by the immunosorbents could not be recovered quantitatively. This failure could be attributed to the inefficacy of the method used for elution but could also be explained by the hypothesis that antibodies were trapped by the three-dimensional polystyrene–antigen conjugates preventing their release into the solution. This problem is further complicated by the heterogeneity of the antigen–antibody systems which was demonstrated in some of the experiments. In accordance with the concept of heterogeneity of antibodies (27), it is possible that during the purification procedure only antibodies that had a low affinity for the antigen or that were formed against minor antigenic components were isolated. Obviously, a combination of these and of some other factors may determine the efficiency of immunologic adsorbents for the isolation and recovery of antibodies in a pure form.

The general features of the present study have been confirmed by Yagi and Pressman (24), who recently reported that with their polystyrene–antigen conjugates the yield of antibody recoveries was of the order of 30 to 35% and that the purity of their antibody preparations approached that found in the present study. Unfortunately it is not possible to evaluate the effect of the molecular weight of the polyamino polystyrene used in their studies, since their starting material was a commercial batch of polystyrene of a not too well-defined molecular weight distribution. In conclusion it can be stated that in spite of the somewhat low yield of precipitating antibodies recoverable by this method, immunosorbents synthesized from polystyrene appear to have the potentiality of leading to the isolation of antibodies in a sufficiently pure form.

Acknowledgments

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THE USE OF POLYSTYRENE–ALLERGEN CONJUGATES FOR THE REMOVAL OF ANTIBODIES FROM SERA OF ALLERGIC INDIVIDUALS[1]

L. Gyenes and A. H. Sehon

Abstract

Polystyrene–ragweed conjugates were shown to remove specifically antibodies from sera of individuals allergic to ragweed. This observation is considered evidence that firm combination occurs in vitro between allergic antibodies and the homologous allergens. Comparative analyses of allergic sera before and after exposure to immunosorbents indicated that complete removal of skin-sensitizing, blocking, and hemagglutinating antibodies did not result in a measurable decrease in protein concentration, thus demonstrating that these factors are present only in minute amounts.

Attempts to elute allergic antibodies from the homologous immunosorbents under various experimental conditions did not lead to their recovery in significant yields; these antibodies could be recovered in small amounts by dissociation with hydrochloric acid at pH 3.

Introduction

It has been shown repeatedly that antibodies in sera of allergic individuals do not precipitate on incubation with the homologous allergens. This failure was attributed to different reasons such as the univalency of these antibodies, their presence in concentrations below the threshold of detectability, or their inability to combine in vitro with the appropriate allergens. Attempts to demonstrate that, in spite of the absence of visible reaction, combination between skin-sensitizing antibodies (reagins) and the corresponding allergens occurred in vitro met with little success except for the variable results obtained with agglutination techniques using collodion particles (1, 2, 3) or tanned red cells coated with the allergens (4, 5, 6). However, in a concurrent study done in this laboratory (7, 8) it was found that all sera of non-treated or treated ragweed-allergic individuals invariably agglutinated red blood cells to which ragweed pollen constituents were coupled via bisdiazotized benzidine. Furthermore, it was established that some parallelism existed between skin-sensitizing and hemagglutinating titers. It was also shown that serum fractions containing skin-sensitizing antibodies (isolated by zone electrophoresis, salting out procedures, and chromatography) were endowed with hemagglutinating capacity (9). Similarly, no separation could be obtained between blocking antibodies and hemagglutinating factors by subjecting sera of treated allergic individuals or of normal volunteers immunized with a water-soluble extract of ragweed pollen to different fractionation procedures.

In the present study an attempt has been made to establish the relationship(s) amongst the different factors present in sera of treated and non-treated allergic individuals sensitive to ragweed.

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Contribution from the Departments of Biochemistry and Chemistry, McGill University, Montreal, Que. The conclusions derived from this study were incorporated in a symposium paper presented at the 3rd Congress of Allergology, Paris 1958, and published in the Proceedings of the Congress, Editions Medicales Flammarion, Paris 1958. Submitted by L. G. to the Department of Biochemistry in partial fulfillment of the requirements for the Ph.D. degree.

Methods and Materials

Preparation of Polystyrene–Allergen Conjugates

The experimental procedures and the materials were similar to those described for the preparation of polystyrene–antigen conjugates (10). For most of the experiments two batches of polyamino polystyrenes were used as starting material, i.e. the highly aminated derivative of PS-2 and the commercial Norsk preparation of polyamino polystyrene. Immunosorbents were prepared by coupling, for each gram of polystyrene, 0.5 g of the water-soluble extracts of ragweed pollen.

Allergic Human Sera

Allergic sera containing skin-sensitizing antibodies were obtained from six non-treated ragweed-sensitive individuals. Sera containing blocking antibodies were collected from three non-allergic volunteers injected subcutaneously with a total of 2,500,000 units* of a water-soluble extract of ragweed pollen (WSR) three times a week over a period of 6 weeks.

Absorption of Sera with Polystyrene–Ragweed Conjugates

The incubation of allergic sera with the appropriate immunosorbents and the recovery of the sera after absorption were performed as described earlier (10). Elution of antibodies from the polystyrene–allergen–antibody complexes was attempted by dissociation with dilute hydrochloric acid at pH 3, with dilute sodium hydroxide at pH 11, with 4 *M* glycine solution, with 5 *M* sodium chloride solution, and with concentrated solutions of the pollen extracts.

The antibody content of the sera (and of the corresponding filtrates and eluates) of non-treated allergic individuals was determined both by the in vitro BDB-hemagglutination technique (8) and by the in vivo passive transfer test of Prausnitz and Küstner (11), referred to hereafter as P–K test. Similarly, the antibody titer of the sera (and of the corresponding filtrates and eluates) obtained from individuals immunized with ragweed extract was established both by the in vitro BDB-hemagglutination technique and by the in vivo blocking test as used in this laboratory (12).

Results

On incubation of sera of ragweed-sensitive individuals with polystyrene–ragweed conjugates, skin-sensitizing and (or) blocking antibodies were removed completely from these sera, as demonstrated by the in vivo P–K and (or) blocking tests. The removal of these antibodies was always accompanied by a parallel disappearance of the hemagglutinating factor(s). These findings were interpreted as evidence that firm combination occurred in vitro between ragweed pollen constituents and skin-sensitizing antibodies, blocking antibodies, and hemagglutinating factor(s).

The specificity of these reactions was demonstrated by a series of experiments similar to those performed with precipitating antigen–antibody systems. When sera containing skin-sensitizing and (or) blocking antibodies were incubated with polystyrene–antigen conjugates prepared with unrelated

*1 unit = 10^{-5} mg protein nitrogen.

antigens, such as NHS, HSA, etc., the skin-sensitizing and (or) blocking titers of the recovered sera did not show any significant decrease. Similar results were obtained using the in vitro hemagglutination technique.

For the physicochemical characterization of the different antibody-like factors in sera of allergic individuals, these sera were analyzed by free electrophoresis and by ultracentrifugation before and after exposure to the homologous immunosorbents. Removal of skin-sensitizing, blocking, and hemagglutinating capacities by absorption with specific immunosorbents did not result in a measurable decrease in protein concentration or in the protein distributions as indicated by the almost identical optical patterns obtained with the sera prior to and after the absorption with polystyrene–ragweed conjugates. From these results it may be concluded that skin-sensitizing and (or) blocking antibodies as well as the hemagglutinating factor(s) were associated with serum components present in concentrations lower than detectable by the Schlieren or the Rayleigh interference fringe systems, i.e. lower than 0.1 mg/ml (13).

Attempts to elute allergic antibodies* from the polystyrene–allergen conjugate with dilute hydrochloric acid at pH 3 did not lead to their recovery in significant yields. On several occasions it was found that the eluates gave threshold P–K reactions in dilutions of only 1:2 or 1:4 as compared to titers of the order of 1000 obtained originally with the whole reaginic sera. Similarly the hemagglutination titers of the eluates were much lower than those of the whole sera. Thus, whilst the whole sera had hemagglutination titers of the order of 500 to 1000, the titers of the eluates ranged from 16 to 32. An analogous trend was observed with sera containing blocking antibodies obtained from normal volunteers immunized with ragweed, i.e. blocking antibodies were recovered from the immunosorbents only in negligible amounts in titers of the order of 2 as compared to titers of about 64 obtained with the whole sera. Correspondingly, the hemagglutination titers of these sera were of the order of 5000, whereas the hemagglutinating factors in the eluates could not be detected in dilutions higher than 1:64.

In order to investigate the reasons for the failure of recovering skin-sensitizing, blocking, and hemagglutinating capacities from the immunosorbents in significant yields the following control experiments were performed.

(i) The reaginic and blocking sera used in this study were treated with hydrochloric acid at pH 3 for 2 hours. Following this treatment it was found that none of the activities of these sera was affected as shown by hemagglutination and by P–K or blocking tests.†

(ii) The same polystyrene–ragweed conjugate was incubated with aliquots of reaginic or blocking sera in successive experiments. In general, a given amount of polystyrene–ragweed conjugate could be used successively for com-

*In the present context skin-sensitizing antibodies and hemagglutinating factors present in sera of allergic individuals, as well as blocking antibodies and hemagglutinating factors found in sera of normal volunteers immunized with ragweed, are lumped together under the term "allergic antibodies".

†However, in other experiments with certain reaginic sera partial or complete loss of skin-sensitizing activity was observed on exposure to an acidic medium.

plete removal of antibodies from two or three equal aliquots of the same serum before becoming saturated with the appropriate antibodies. Once saturation was reached, no further removal of antibodies from a fresh sample of serum could be effected with the same portion of polystyrene–ragweed conjugate. This indicates that disappearance of skin-sensitizing and hemagglutinating activities from allergic sera on exposure to polystyrene–ragweed conjugates was due to the specific removal and not to the inactivation of these factors by the immunosorbent.

(iii) Attempts to elute antibodies from the immunosorbents by dissociation with sodium hydroxide, sodium chloride, or glycine were unsuccessful not only with sera containing skin-sensitizing and (or) blocking antibodies but also with strong rabbit antisera containing precipitating antibodies to ragweed pollen constituents.

(iv) Rabbit antiragweed sera in dilutions as high as 1:4000 were shown to block the reaction between skin-sensitizing antibodies and the allergen. However, the rabbit antiragweed antibodies eluted from polystyrene–ragweed conjugates, by dissociation at pH 3, were capable of blocking the P–K test only in dilutions up to 1:200.

From the results of these experiments it may be concluded that the failure to recover quantitatively skin-sensitizing and blocking antibodies as well as hemagglutinating factors from the immunosorbents has to be attributed to the inadequacy of the elution methods used or to possible denaturation of these moieties on the surface of the immunosorbent.

Discussion

Although polystyrene–allergen conjugates were capable of removing specifically antibodies from allergic sera, these antibodies could not be eluted in significant quantities from the immunosorbents. Nevertheless, on the basis of the results obtained, several conclusions can be drawn concerning the nature of the different antibody-like factors present in allergic sera.

The removal of antibodies by the appropriate immunosorbents may be considered as evidence that skin-sensitizing and blocking antibodies as well as the hemagglutinating factor(s) combine firmly in vitro with the homologous allergens. The results of electrophoretic analyses of allergic sera before and after absorption with the appropriate immunosorbents indicated that the factors responsible for skin-sensitization, blocking of the P–K reaction, and hemagglutination must be present in exceedingly low concentrations. These findings are in good agreement with a previous observation made in this laboratory (14) that maximum skin-sensitizing activity was often associated with serum fractions separated by zone electrophoresis, which had an extremely low protein concentration (0.8 mg protein/ml or less).

As mentioned earlier, allergic sera containing skin-sensitizing and (or) blocking antibodies invariably agglutinated red blood cells to which ragweed or grass pollen constituents were coupled via bisdiazotized benzidine (8, 15). The skin-sensitizing and hemagglutinating titers of allergic sera or of serum fractions were not identical, nor was there a simple relationship between these

two factors, but on the whole one may say that some parallelism existed between skin-sensitizing and hemagglutinating titers (9).* In the present study it was found that some parallelism existed between skin-sensitizing and hemagglutinating titers both with allergic sera and with antibody preparations eluted from the immunosorbents by dissociation at pH 3. Furthermore, it was found that absorption of allergic sera with an insufficient amount of immunosorbent resulted only in partial, but parallel, depletion of both skin-sensitizing and hemagglutinating factors. From these results one may be led to conclude that both skin-sensitizing and blocking antibodies possess also hemagglutinating capacity, and that the in vitro hemagglutination reaction may be used for detection of skin-sensitizing and blocking antibodies. The alternate interpretation that skin-sensitizing antibodies may be independent moieties from hemagglutinating factors found in sera from non-treated allergic individuals, and that blocking antibodies may be separate entities from hemagglutinating factors in sera of treated individuals, cannot be ruled out. Obviously, to settle this problem one would require further knowledge of the true nature of the factors responsible for skin-sensitizing, blocking, and hemagglutinating abilities.

Acknowledgments

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*It ought to be pointed out that there is no reason for expecting identical titers with the in vivo and hemagglutinating methods, since each method has its own limit of sensitivity.

IRRADIATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE IN DILUTE AQUEOUS SOLUTIONS[1]

D. K. MYERS

Abstract

In extension of previous in vivo experiments, the effects of X irradiation on DPN were studied in vitro. No correlation between the effects on the ultraviolet absorption spectrum and on the coenzyme function of DPN was evident after irradiation at different pH values. However, the loss of coenzyme function could be correlated with the destruction of ribose and of riboside linkages. Catalase did not provide greater protection than did other proteins. It was concluded that the observed loss of DPN from irradiated cells is not due to the radiosensitivity of the DPN molecule itself.

Introduction

After exposure of rats to high doses of X rays, the amount of diphosphopyridine nucleotide (DPN; coenzyme I) in the liver, spleen, and thymus is diminished (1). In order to determine the mechanism of this effect, the destruction of DPN by irradiation was studied in vitro.

Barron *et al.* (2) reported that relatively high doses of X radiation are required to produce an appreciable change in the ultraviolet absorption spectrum of DPN in dilute aqueous solutions. However, the absorption spectrum is due mainly to the adenine portion of the DPN molecule; the coenzyme function of DPN, which requires the intact molecule, might be more radiosensitive. This supposition was readily confirmed. The kinetics and mechanism of the destruction of the coenzyme function of DPN by irradiation were, therefore, investigated in more detail.

Methods

In most experiments, 5×10^{-5} *M* or 10^{-4} *M* solutions of DPN were prepared in aerated 0.01 *M* potassium phosphate buffer at pH 7.5. Organic buffers, e.g. 10^{-3} *M* sodium acetate, were unsuitable since they protected DPN against the effects of irradiation. The solutions were irradiated at $20\pm1°$ C at a distance of 33 cm from the target of a 2000-kv X-ray machine operated at 1.5 ma; the dose rate, measured in air, was 1600 r/minute in all experiments.

The optical densities of the solutions in the ultraviolet region were measured in a Beckman DU spectrophotometer. The coenzymic activity of DPN was determined by reaction with ethanol in the presence of purified alcohol dehydrogenase (3). Ribose was measured by the orcinol reaction (4), and free adenine by oxidation to dihydroxyadenine in the presence of purified xanthine oxidase (5). The nicotinamide–riboside linkage can be estimated conveniently from the increase in extinction at 325 mμ on addition of cyanide to the DPN solution (3). For this purpose, 2 ml of DPN solution was mixed (*a*) with 1 ml 0.4 *M* Na_3PO_4 at pH 11.5, and (*b*) with 1 ml 0.4 *M* Na_3PO_4 containing 3 *M* KCN;

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nicotinamide–riboside was calculated from the difference in the optical densities of these two solutions.

DPN (>95% purity), reduced DPN, and hexokinase were obtained from Pabst Laboratories, Milwaukee; nicotinamide ribotide (nicotinamide mononucleotide) from Sigma Chemical Co., St. Louis; purified alcohol dehydrogenase, xanthine oxidase, bovine serum albumin, and catalase from Nutritional Biochemicals Corp., Cleveland. A second sample of crystalline catalase was supplied by Worthington Biochemical Corp., Freehold, N.J.

Results

Kinetics of the Destruction of the Coenzyme Function of DPN

The concentration of unaltered DPN decreased along an exponential curve as the dose of radiation was increased; when the logarithm of DPN concentration was plotted against dose, a straight line was obtained (Fig. 1). The slope

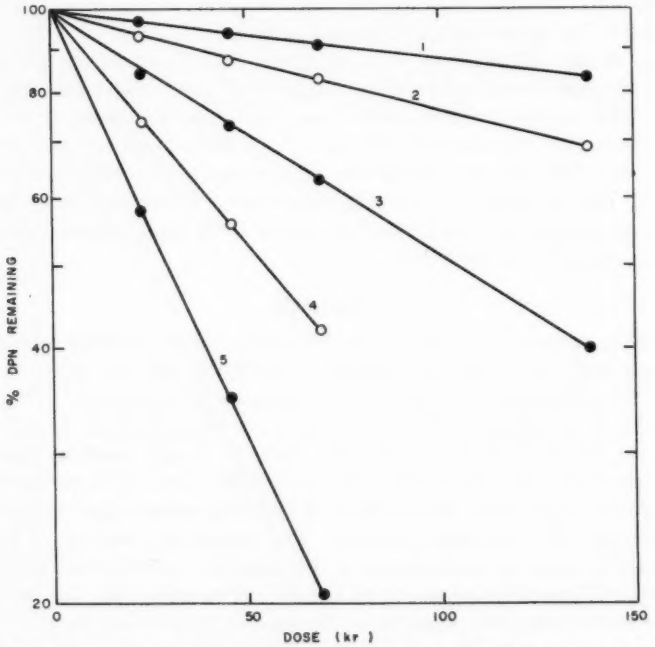

FIG. 1. Destruction of coenzymic activity of DPN by irradiation in 0.01 *M* phosphate at pH 7.4.
(1) 10×10^{-4} *M* DPN; D_{37} = 750 kr.
(2) 5×10^{-4} *M* DPN; D_{37} = 380 kr.
(3) 2×10^{-4} *M* DPN; D_{37} = 150 kr.
(4) 1×10^{-4} *M* DPN; D_{37} = 79 kr.
(5) 0.5×10^{-4} *M* DPN; D_{37} = 43 kr.

of this line was inversely proportional to the initial concentration of DPN in the solution before irradiation, provided this initial concentration was greater

than 10^{-4} *M* (Fig. 1). This is the result to be expected if the products formed by irradiation of DPN react as readily as DPN itself with the free radicals produced in the irradiated solution (6, 7). The efficiency of the reaction decreased when the initial concentration of DPN was less than 10^{-4} *M* (Fig. 1); similar results have been observed with many other compounds and are usually ascribed to recombination of the free radicals in the irradiated solution before they reach the substrate molecule (7).

Control experiments were carried out to determine whether the temperature and the presence of phosphate buffer during irradiation influenced the rate of destruction of DPN appreciably (Table I). Phosphate appeared to do so but only when present in concentrations above 0.01 *M*. Variations in temperature between 2° and 41° C had little effect on the rate of the reaction.

TABLE I

Effect of phosphate concentration and temperature on the radiosensitivity of 5×10^{-5} *M* DPN at pH 7.4

Concentration of potassium phosphate (*M*)	Temperature (°C)	Calculated dose for 63% loss of DPN activity (kr)
0.05	21	53
0.02	21	47
0.01	21	44
0.002	21	42
0.0005	21	43
0.0	21	42
0.01	2	43
0.01	21	44
0.01	41	49

Oxidation of DPNH

Irradiation of reduced DPN (i.e., DPNH) in aqueous solution results in an oxidation to DPN (2) which can be followed conveniently by the disappearance of the absorption maximum at 340 mμ. The initial decrease in the extinction of DPNH at 340 mμ (E_{340}) was directly proportional to the dose of radiation (Fig. 2). Subsequently the rate of oxidation of DPNH was retarded, presumably due to accumulation of DPN, which would compete with the residual DPNH for free radicals in the irradiated solution. As the value of E_{340} decreased, the absorption maximum at 260 mμ increased slightly (Fig. 2). This result agrees with the conclusion (2) that the initial effect of radiation is predominantly the oxidation of DPNH to DPN; complete oxidation of DPNH should increase the value of E_{260} by approximately 12% (8). Under the same experimental conditions, DPN was found to be 2.1 times less sensitive than DPNH to the effects of radiation (cf. Figs. 1 and 2).

Changes in the Ultraviolet Absorption Spectrum of DPN

On irradiation of 5×10^{-5} *M* DPN in 0.01 *M* phosphate buffer at pH 7.4, the initial changes in the ultraviolet absorption spectrum were qualitatively identical with those described by Barron *et al.* (2) in the case of adenine, notably a decrease in the absorption maximum at 260 mμ and an increase in the mini-

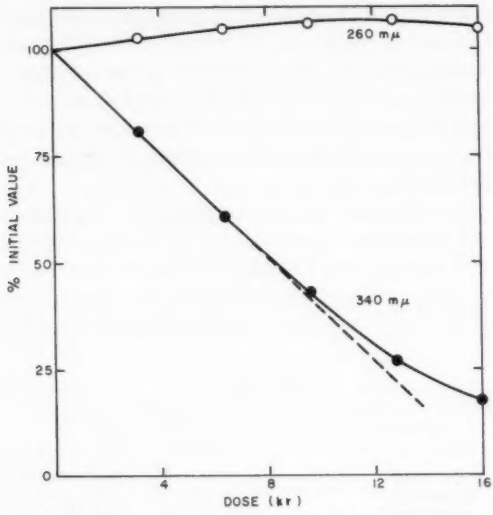

FIG. 2. Changes in the absorption maxima of 4×10^{-5} *M* DPNH during irradiation at pH 7.4.

mum near 230 mμ. At pH 7.4, the value of E_{260} appeared to decrease slowly along an exponential curve as the dose of radiation increased; however, at pH 4.5, the initial decrease in E_{260} for DPN was directly proportional to the dose of radiation (Fig. 3). Following Lea's procedure for estimation of relative rates

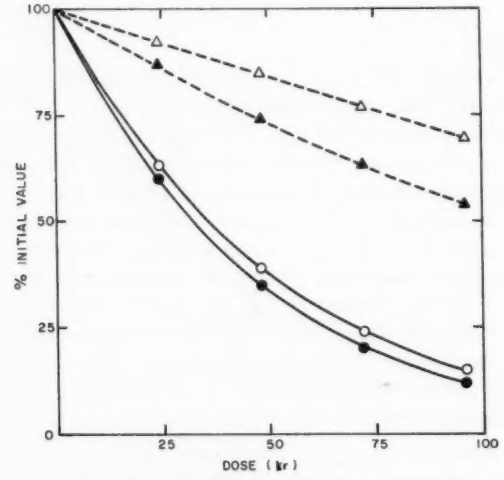

FIG. 3. Destruction of 5×10^{-5} *M* DPN during irradiation.
pH 7.4, coenzyme function (●).
pH 4.5, coenzyme function (○).
pH 7.4, E_{260} (▲).
pH 4.5, E_{260} (△).

of destruction, 63% decrease extrapolated on an exponential curve has been compared with 100% decrease extrapolated on a linear plot (7, 9). This comparison indicated that a change in pH from 7.4 to 4.5 diminished the relative radiosensitivity of the E_{260} value by a factor of two (Table II). The rate of destruction of the coenzyme function of DPN, on the other hand, was scarcely affected by alterations in pH between 7.4 and 4.5. Thus the ratio of these two parameters varied from approximately 3 to 8, depending on the pH of the DPN solution during irradiation (Table II).

TABLE II

Effect of pH on the radiosensitivity of 5×10^{-5} *M* DPN

Buffer			Calculated dose for 63% loss of DPN activity (kr)		
Compound	Concn. (*M*)	pH	(*a*) Coenzyme function	(*b*) E_{260}	Ratio (*b*)/(*a*)
Potassium phosphate	0.01	8.4	43	142	3.3
Potassium phosphate	0.01	7.4	46	155	3.4
Potassium phosphate	0.01	5.9	48	—	—
Potasiusm phosphate	0.01	4.5	50	310*	6.2
H_2SO_4	0.0005	3.2	53	385*	7.3
HCl	0.001	2.9	64	—	—
H_2SO_4	0.01	2.0	110	900*	8.2
HCl	0.01	1.9	152	975*	6.4

*Calculated dose for 100% decrease on a linear plot (cf. Fig. 2).

Since the adenine group is responsible for most of the E_{260} value of DPN, the above results suggested that alterations in the adenine rings could not account for all the loss of coenzyme activity in irradiated solutions of DPN, particularly at low pH values. The nicotinamide ring, which accounts for the remainder of the E_{260} value, appeared to be even more resistant to the effects of irradiation. In one experiment, 10^{-4} *M* solutions of DPN and of nicotinamide ribotide in 0.01 *M* phosphate at pH 7.4 were exposed to 96 kr; this exposure resulted in 70% loss of the coenzyme function of DPN and 32% decrease in E_{260} for DPN but only 18% decrease in the absorption maximum of nicotinamide ribotide at 265 mμ.

Destruction of Ribose and Riboside Linkages in DPN

Both ribose groups in the DPN molecule are altered by irradiation in a manner which renders them unreactive in the orcinol test for pentoses. As in the case of the coenzymic activity of DPN, the decrease in total ribose appeared to follow an exponential curve during irradiation of DPN solutions. The ratio of the doses required to reduce the total ribose and coenzymic functions to 37% of their initial values remained constant at 2.6–2.7, both in neutral and acid solutions of DPN (Table III). By varying the acid concentration and heating time employed in the orcinol test, it was possible to reduce the contribution of the adenine–ribose group from 50% to 29% of the total color developed in this test (10). However, this variation in the test for ribose did not

TABLE III

Rates of destruction of total ribose and of nicotinamide–riboside bond during irradiation of 10^{-4} *M* DPN

pH	Dose required to produce 63% decrease (kr)			
	Coenzymic activity	Total ribose	Nicotinamide–riboside bond	E_{260}
9.4	66	—	152	214
7.4	78	201	184	268
4.3	86	227	216	550*
1.9	268	735	735	1800*

*Calculated dose for 100% decrease on a linear plot (cf. Fig. 2).

alter the percentage destruction observed following irradiation. In other words, both ribose groups in the DPN molecule must be destroyed at approximately the same rate by irradiation.

Scholes and Weiss (11) have concluded that a small portion of the destruction of adenosine and derivatives by irradiation is due to hydrolysis of the adenine–riboside linkage following oxidation of the ribose group. An analogous but larger destruction of the nicotinamide–riboside linkage was evident after irradiation of DPN solutions. The amount of nicotinamide–riboside decreased during irradiation in much the same manner as total ribose (Table III).

A specific method for a parallel assay of the adenine–riboside linkage was not available. However, destruction of this bond could be demonstrated by an assay for free adenine liberated from the DPN during irradiation (Fig. 4). Since free adenine in the solution is also destroyed during irradiation (2), the adenine concentration decreased again as the irradiation was prolonged (Fig. 4). However, the rate of hydrolysis of the adenine–riboside linkage during irradiation can be estimated from the initial rate of liberation of adenine during the time that a large excess of DPN is present in the solution. For example, 4.8 and 9.2×10^{-6} *M* concentrations of free adenine were found after exposure of a 10^{-4} *M* DPN solution at pH 7.4 in 0.01 *M* phosphate to 8 and 16 kr, respectively (Fig. 4). Assuming that the destruction of the adenine–riboside linkage follows an exponential curve, then the dose required for 63% destruction would be approximately 164 kr. On this basis, the adenine–riboside and nicotinamide–riboside linkages would appear to be almost equally sensitive to the effects of irradiation (cf. Table III). This conclusion was confirmed by analysis of 10^{-4} *M* DPN solutions irradiated at pH 1.9, where free adenine appeared to be relatively resistant to the effects of irradiation; after exposure to 96 kr, 12.0% of the nicotinamide–riboside linkage had disappeared and 12.2% of the total adenine could be recovered as free adenine in the solution.

Effect of Catalase and Other Proteins

Barron *et al.* (2) reported that purine and pyrimidine derivatives were protected against the effects of irradiation by addition of catalase to the solution. Since it has also been reported that hydrogen peroxide is as effective as radiation in causing a loss of DPN from living cells (12), the effect of catalase on the destruction of DPN by irradiation in vitro was studied in more detail. It

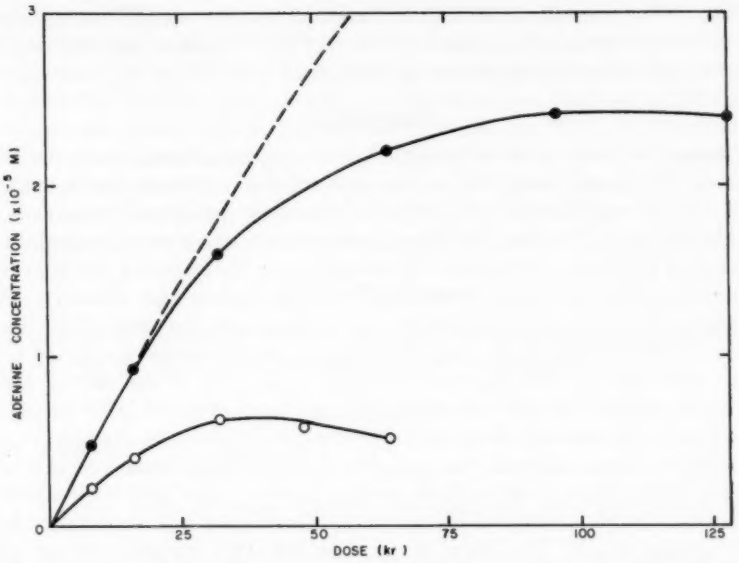

FIG. 4. Concentrations of free adenine found in DPN solutions after irradiation at pH 7.4: 5×10^{-5} *M* DPN (○); 10^{-4} *M* DPN (●); theoretical curve for the liberation of adenine from 10^{-4} *M* DPN with no simultaneous destruction of adenine (– – –).

appeared that high concentrations of catalase were required to provide appreciable protection to the DPN. Furthermore, the effect of catalase was not decreased in the presence of potassium cyanide, a catalase inhibitor, and was duplicated by other proteins (Table IV). Apparently the protective action of catalase is not dependent on its ability to decompose hydrogen peroxide.

TABLE IV

Effect of catalase and other proteins on the destruction of 5×10^{-5} *M* DPN during exposure to 40 kr at pH 7.4

Added protein	Protein concentration (mg/ml)	Decrease in DPN activity (%)	
		No cyanide	With 10^{-3} *M* cyanide
None	0.0	60	47
Catalase A	0.2	39	28
Catalase B	0.2	38	28
Catalase B	1.0	15	11
Albumin	0.2	41	30
Albumin	1.0	16	—
Hexokinase	0.2	18	—

The protective action of proteins is important for a comparison of the radiosensitivity of DPN in vitro and in vivo. In an attempt to approach more closely to conditions in vivo, a 2×10^{-4} *M* solution of DPN in 0.01 *M* phosphate buffer at pH 7.4 was irradiated in the presence of 100 mg serum albumin/ml

solution. After exposure to 96 kr, the coenzymic activity of DPN was reduced by 7%; larger decreases in the amount of DPN in spleen and thymus are observed after exposing an animal to as little as 8 kr (1).

Discussion

Although we have not investigated all of the possible alterations in the DPN molecule, the results show that a large part of the inactivation of coenzymic activity can be explained by destruction of the ribose groups and rupture of the riboside linkages. The data on rates of ribose destruction must necessarily be regarded as minimum values since we do not know the extent of the alteration required to render the ribose group inactive in the orcinol test. However, even these minimum rates of ribose destruction could account for most of the loss of DPN in irradiated solutions. As noted above, both the ribose groups of DPN appear to be destroyed at approximately the same rate. If the destruction of each ribose group proceeds independently, the proportion of DPN molecules with at least one oxidized ribose group will be greater than the average proportion of total ribose destroyed. For example, if $X\%$ of the adenine–ribose groups and $X\%$ of the nicotinamide–ribose groups remain intact after exposure to a given dose of radiation, then $X^2\%$ of the DPN molecules will remain with both ribose groups intact. The slope of the line obtained by plotting log (X^2) against dose will, of course, be double the slope of log X/dose. Consequently the dose required for 63% inactivation of 10^{-4} M DPN due to ribose destruction alone should have a maximum value of 101 kr, rather than 201 kr (Table III), at pH 7.4. Under the same conditions, 86 kr should be sufficient to destroy at least one of the two riboside linkages in 63% of the DPN molecules; this value is only 10% higher than the measured value, 78 kr, required to destroy 63% of the coenzymic activity of DPN (Table III). The discrepancy between these two values must be due to destruction of the adenine group and other portions of the DPN molecule by irradiation.

A comparison of the relative radiosensitivities of different compounds in aqueous solution should apparently be carried out at a specified pH; this precaution has not always been observed in previous investigations. The explanation of the observed pH effects (Fig. 3 and Table II) is still uncertain. One factor may be alterations in the structure of the substrate, e.g., the free amino group on the adenine moiety becomes ionized as the pH is reduced below 4 (13). Another factor may be the relative yields of the different products formed by irradiation of water, e.g., the yield of hydrogen peroxide tends to increase in acid solutions (14).

It is highly improbable that the radiosensitivity of the DPN molecule itself could account for the observed loss of DPN following irradiation of mammalian cells (1, 15). For example, a dose of 8 kr will have little effect on DPN in dilute aqueous solutions when the concentration of DPN is above 10^{-4} M (Fig. 1) and the presence of protein will provide considerable protection under these conditions (Table IV). However, the spleen and thymus of rats, which contain approximately $2\text{–}3\times10^{-4}$ M DPN on the average, lose 50% of their DPN on exposure to 8 kr (1). Furthermore, the destruction of DPN by irradiation in

vitro does not appear to involve hydrogen peroxide (Table IV), while the effects of irradiation on the DPN content of suspensions of tumor cells (15) can be duplicated by the addition of equivalent amounts of hydrogen peroxide to the suspending medium (12). A possible explanation for the loss of DPN from irradiated cells is provided by the existence of an enzyme which rapidly destroys DPN in tissue homogenates (16). Presumably DPN is not normally exposed to the action of this enzyme in living cells; however, irradiation might release either this enzyme or the DPN from its bound state in the cell, in the same manner as ribonuclease (17) and acid deoxyribonuclease (18) are released.

Acknowledgment

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THE INFLUENCE OF ADRENALECTOMY UPON THE ACTIVITY OF THE HEXOSEMONOPHOSPHATE SHUNT IN THE LIVERS AND MAMMARY GLANDS OF LACTATING RATS[1]

J. S. WILLMER

Abstract

The development of the hexosemonophosphate shunt in mammary tissue and liver of lactating rats has been studied. A sixfold increase in mammary glucose-6-phosphate dehydrogenase levels between parturition and weaning was accompanied by a considerable increase in 6-phosphogluconate dehydrogenase activity. The hepatic level of the former enzyme was also elevated 11-fold during this period. Adrenalectomy at parturition, or on the 3rd, 6th, 9th, or 14th days of lactation, depressed the activity of this pathway in mammary gland, a lowered level being observed in all cases after operation. A slight increase in enzyme activity was found in hepatic tissue in the immediate postoperative period; this was succeeded by a decrease.

These results are discussed in relation to the growth changes observed in groups of unoperated and adrenalectomized rats.

Introduction

The influence of adrenalectomy upon lactation has not been clearly elucidated. Milk secretion is presumably reduced following adrenalectomy, judging by the subnormal weight gains of suckling rats (1, 2), which result in part from reduced food intake but mainly from the fact that the adrenal glands are essential for a normal secretory process. Folley and Greenbaum (3) studied changes in arginase and alkaline phosphatase activity in rat mammary gland and liver during pregnancy, lactation, and involution. They observed that the normal increase in activity of mammary gland and liver arginase during lactation was inhibited when adrenalectomy was performed on the third day *post partum*. There was no significant effect on mammary gland phosphatase (4). Folley and Watson (5) were able to demonstrate that replacement therapy with cortisone was more effective than desoxycorticosterone in restoring arginase activity, though neither of these steroids was thought to restore fully milk production or litter weight increase.

Suggestions have been made which link the direct oxidative pathway of glucose metabolism, or hexosemonophosphate shunt, with the synthesis of nucleic acids and proteins (6) and with lipogenesis (7). It has also been demonstrated that a considerable portion of the glucose catabolized by the lactating mammary gland is utilized by this pathway (8). Glock and McLean (9) traced the development of enzymic activity in mammary gland tissue and found that between the end of pregnancy and weaning the activity was increased 60-fold for glucose-6-phosphate dehydrogenase and 20-fold for 6-phosphogluconate dehydrogenase. It is of interest to speculate upon the possible association of this pathway, during a period of rapid metabolic activity, with either the development of glandular tissue or with the synthesis of milk constituents.

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This study was therefore undertaken to observe the effect of adrenalectomy at various stages of lactation upon the activity of the hexosemonophosphate shunt in liver and mammary gland. This was correlated with the rate of development of the offspring of adrenalectomized rats.

Methods

Primiparous albino rats of the Wistar strain, age 3½ to 4 months, and weight 200–250 g, were used in these investigations. All litters were reduced to four animals at parturition. Bilateral adrenalectomy by the dorsal approach was performed under Nembutal anaesthesia in groups of rats at parturition, and on the 3rd, 6th, 9th, and 14th days of lactation. During the postoperative period adrenalectomized animals had free access to fox cubes and 1% sodium chloride solution. Unoperated and sham-operated rats were placed under similar conditions, but received tap water instead of saline.

Enzyme assays were performed in most cases 3 days after adrenalectomy and on the 19th day of lactation. In the case of animals adrenalectomized at parturition enzyme levels were determined on the third and fifth day of lactation.

The method of Glock and McLean (10) was used to estimate glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity. The rate of reduction of TPN in the presence of glucose-6-phosphate or 6-phosphogluconate as substrates was measured spectrophotometrically at 340 mμ during a 5-minute period. As the influence of adrenalectomy upon milk composition was not known it was felt inadvisable to adopt any of the accepted methods of correction for the milk content of mammary tissue. Instead, the material removed from the abdominal glands was cut into small pieces, allowed to exude milk at 0° C, and then blotted as dry as possible. This method gave very reproducible results under the conditions investigated. In the case of mammary tissue the supernatant fluid from 5% or 2.5% homogenates, depending upon the stage of lactation, gave satisfactory rates of reduction of TPN. In the case of liver, supernatant fluids from 5% homogenates were always used. A unit of activity is defined as the quantity of enzyme which reduces 0.01 μmole TPN/minute at 20° C.

Growth curves given in Fig. 1 are composites of all suckling animals used in the tests, the growth of these animals being regarded as normal until the day their dams were adrenalectomized. In Figs. 2–4 the smooth curves for activity of liver and mammary gland enzymes represent the means from groups of three to six adult rats.

Results and Discussion

Growth data for the litters of control and adrenalectomized rats are presented in Fig. 1. Sham operation was not found to cause statistically significant changes in litter growth or tissue enzyme levels and these results have not been incorporated in the tables. Mortality was high among litters from rats adrenalectomized at parturition and on the third day of lactation. In the latter case litter weight changes were recorded until the 19th day of lactation and it

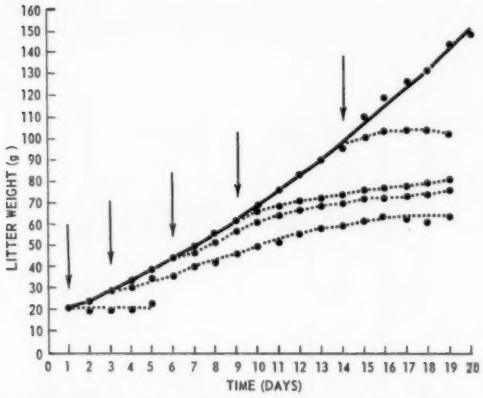

FIG. 1. Weight changes in litters from adrenalectomized and unoperated lactating rats. (All litters reduced to four animals at parturition.) ●——● Litters from unoperated animals. ●- - -● Litters from adrenalectomized animals. ↓ Indicates time of adrenalectomy.

may be that the results tabulated for survivors should not be considered truly representative. Rats adrenalectomized at parturition were unable to maintain their litters beyond the fifth day of lactation.

It is evident that, at whatever stage of lactation adrenalectomy was performed, litter growth rate was depressed following the operation. Adrenalectomy prior to the sixth day of lactation caused a high mortality rate among litters. When adrenalectomy was performed after the sixth day of lactation suckling rats generally survived until weaning, although litter weights were reduced.

The reduced growth rate shown by the litters of adrenalectomized rats is reflected in the changes observed in the level of activity of the enzymes of the hexosemonophosphate shunt in the mammary gland (Tables I and II; Figs. 2 and 3). Glucose-6-phosphate dehydrogenase activity dropped sharply in all groups of rats during the immediate postoperative period; this was most pronounced in the group adrenalectomized on the 14th day of lactation. In those groups adrenalectomized at parturition and on the third day of lactation the enzyme activity dropped to the level present in the involuted gland, in which milk secretion has ceased, which probably explains the high mortality rate in these groups. Females adrenalectomized on the 6th, 9th, or 14th days of lactation exhibited a further decrease in glucose-6-phosphate dehydrogenase levels on day 19, but in all these groups the activity was maintained at a higher level than in the involuted gland. Similar changes were observed with 6-phosphogluconate dehydrogenase (Fig. 3).

The characteristic changes in the activity of these enzymes in control animals during lactation and involution follows very closely the distribution described by Glock and McLean (8). The values obtained at parturition for both enzymes were, however, considerably higher than found by these authors, as also were the involutionary levels.

TABLE I

Activity of mammary glucose-6-phosphate dehydrogenase in normal and adrenalectomized lactating rats*

Treatment	Day of lactation									
	1	3	5	6	9	12	14	17	19	24
Unoperated controls†	699 ±43	903 ±67		1703 ±90	2108 ±247		3153 ±40		4032 ±117	456 ±95
Adrenalectomized on 1st day of lactation		476 ±105	429 ±115							
Adrenalectomized on 3rd day of lactation				742 ±61					452 ±39	
Adrenalectomized on 6th day of lactation					1218 ±230				1048 ±77	
Adrenalectomized on 9th day of lactation						1702 ±158			1258 ±168	
Adrenalectomized on 14th day of lactation								1819 ±134	1472 ±205	

*Enzyme activity expressed as units per gram fresh tissue ± standard deviation.
†Results with sham-operated controls statistically similar.

TABLE II

Activity of mammary 6-phosphogluconate dehydrogenase in normal and adrenalectomized lactating rats*

Treatment	Day of lactation									
	1	3	5	6	9	12	14	17	19	24
Unoperated controls†	214 ±14	220 ±41		258 ±47	332 ±39		533 ±124		710 ±65	207 ±48
Adrenalectomized on 1st day of lactation		186 ±37	144 ±33							
Adrenalectomized on 3rd day of lactation				200 ±18					181 ±24	
Adrenalectomized on 6th day of lactation					210 ±23				177 ±57	
Adrenalectomized on 9th day of lactation						291 ±49			242 ±72	
Adrenalectomized on 14th day of lactation								359 ±37	250 ±32	

*Enzyme activity expressed as units per gram fresh tissue ± standard deviation.
†Results with sham-operated controls statistically similar.

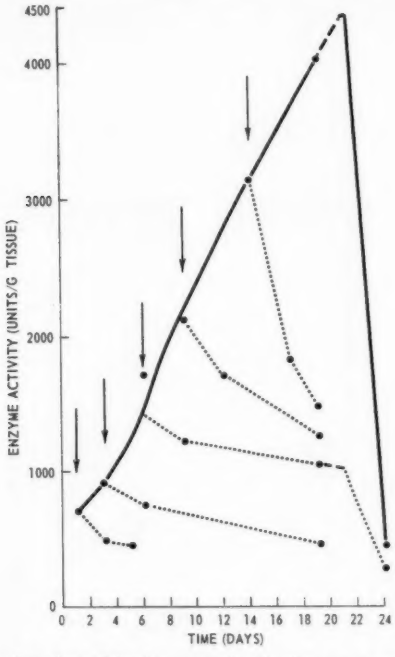

FIG. 2. Glucose-6-phosphate dehydrogenase levels of mammary glands from adrenalectomized and unoperated lactating rats.
●——● Unoperated animals. ●– – –● Adrenalectomized animals. ↓ Indicates time of adrenalectomy. ●— —● Extrapolated value.

Simultaneous studies were made of changes occurring in the activity of the direct oxidative pathway in the livers of lactating adrenalectomized and control rats (Table III; Fig. 4). A steady increase in activity of glucose-6-phosphate dehydrogenase was observed between parturition and weaning in unoperated animals, followed by a sharp drop corresponding to the involutionary period. These changes are similar to those occurring in the mammary gland where adrenalectomy was observed to depress the normal increase in activity, irrespective of the stage of lactation, and in some cases to cause a pronounced loss of enzymatic activity by the 19th day. However, the activity of glucose-6-phosphate dehydrogenase in liver was found to increase during the immediate postoperative period, except in the groups adrenalectomized on the 14th day. It has been shown that the activity of hepatic 6-phosphogluconate dehydrogenase does not always parallel the changes occurring in glucose-6-phosphate dehydrogenase levels (11). In the present series of investigations, hepatic 6-phosphogluconate dehydrogenase activity varied from approximately 350 to 550 units/g tissue. There was no correlation between the changes in the activity of this enzyme and glucose-6-phosphate dehydrogenase activity, the development of lactation, or the influence of adrenalectomy.

Folley and Greenbaum (3) have shown that whereas milk secretion increases

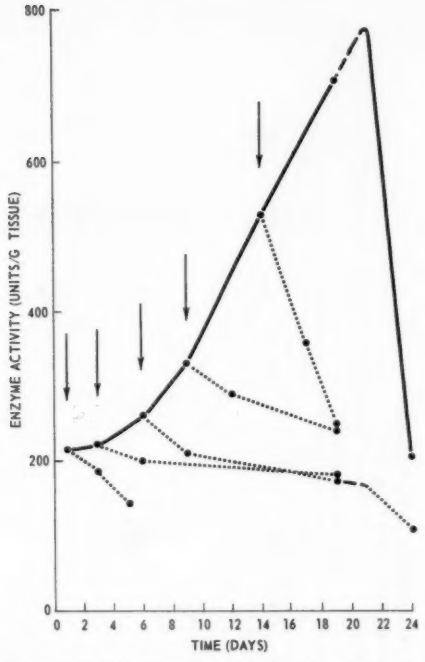

FIG. 3. 6-Phosphogluconate dehydrogenase levels of mammary glands from adrenalectomized and unoperated lactating rats.
●——● Unoperated animals. ●- - -● Adrenalectomized animals. ↓ Indicates time of adrenalectomy. ●— —● Extrapolated value.

throughout the period of lactation, this is not accompanied by a corresponding increase in the weight of glandular tissue. It would appear therefore that the elevated level of arginase (3) and the increased activity of the hexosemonophosphate shunt observed in mammary glands from lactating rats are probably implicated directly in the synthesis of milk constituents. The functional importance of the direct oxidative pathway of glucose metabolism is a matter of conjecture, and it has not been unequivocally shown to participate in any of the biosynthetic processes associated with lactogenesis. However, the greatly increased activity of this pathway in mammary gland during lactation, higher than in any other mammalian tissue examined, is indicative of its association with milk formation. The concomitant increase in the hepatic level of this pathway might be a purely adaptive change due to a generally elevated metabolic rate. It is possible, however, that the elevated level of glucose-6-phosphate dehydrogenase in the liver is concerned with the supply of metabolites for use in the biosynthesis of milk constituents.

While some difference of opinion exists as to the efficacy of individual adrenal steroids in restoring lactation to normal in adrenalectomized rats (12–14) preliminary investigations in this laboratory have shown that a daily dose of 3 mg cortisone will promote normal growth in the litters of rats adrenalectom-

TABLE III

Activity of hepatic glucose-6-phosphate dehydrogenase in normal and adrenalectomized lactating rats*

Treatment	Day of lactation									
	1	3	5	6	9	12	14	17	19	24
Unoperated controls†	110 ±8	133 ±8		271 ±15	392 ±19		668 ±9		1064 ±57	597 ±80
Adrenalectomized on 1st day of lactation		173 ±20	168 ±9							
Adrenalectomized on 3rd day of lactation				185 ±11					142 ±19	
Adrenalectomized on 6th day of lactation					313 ±10				278 ±56	
Adrenalectomized on 9th day of lactation						473 ±120			315 ±89	
Adrenalectomized on 14th day of lactation								522 ±36	400 ±48	

*Enzyme activity expressed as units per gram fresh tissue ± standard deviation.
†Results with sham-operated controls statistically similar.

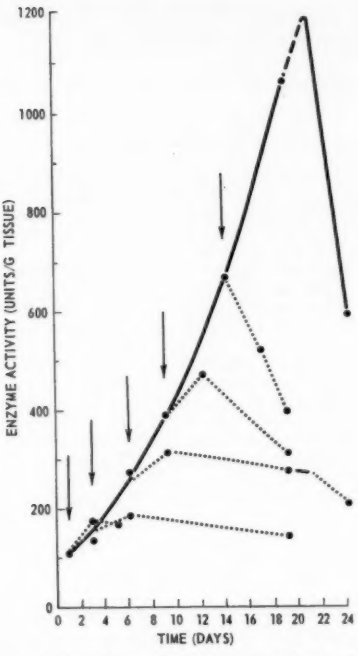

FIG. 4. Hepatic glucose-6-phosphate dehydrogenase levels in adrenalectomized and unoperated lactating rats.
●——● Unoperated animals. ●- - -● Adrenalectomized animals. ↓ Indicates time of adrenalectomy. ●— —● Extrapolated value.

ized on the sixth day of lactation. This is accompanied by development of the hexosemonophosphate shunt in mammary gland and liver at a rate indistinguishable from control animals.

Acknowledgments

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THROMBOCYTOPENIA IN THE EXPERIMENTAL PRODUCTION OF HEMORRHAGIC DEATH BY MULTIPLE FACTORS[1]

F. R. CALARESU[2] AND L. B. JAQUES

Abstract

Three experimental conditions were established in rats either singly or in combination: (*a*) thrombocytopenia by the intraperitoneal administration of a single dose of radioactive phosphorus, 4 μc/g of body weight, (*b*) prothrombopenia by the oral administration of dicumarol, 2 mg/100 g of body weight per day for 7 days, (*c*) stress by the intraperitoneal administration of a single dose of 10% NaCl, 2.5 ml/100 g of body weight. The response to the treatment was measured by the level of circulating platelets and the prothrombin time. The cause of death was assessed by gross and, occasionally, histological examination of the organs of the animals that had died within 15 days of the injection of the radioactive phosphorus.

The percentage mortality from spontaneous hemorrhage when thrombocytopenia was combined with either hypocoagulability or stress was very much greater than the mortality resulting from a single treatment, or from a simple additive effect ($P \ll 0.01$). Mortality due to hemorrhage was thrombocytopenia, 20%; P^{32} without thrombocytopenia, 0% (also together with dicumarol and with stress); dicumarol, 16%; stress, 0%; thrombocytopenia and dicumarol, 81%; thrombocytopenia and stress, 67%; controls, 0%. These results provide additional support for the hypothesis of spontaneous hemorrhage being caused by multiple factors acting simultaneously.

Introduction

In the whole animal there are at least three interrelated mechanisms (1, 2, 3) which maintain hemostasis or prevent blood loss from spontaneous hemorrhage: (1) platelet massing, (2) formation of fibrin, (3) integrity of the blood vessel wall. Jaques and co-workers (4, 5) have shown that in animals to which anticoagulants are administered spontaneous hemorrhage occurs when the animals are subjected to various stress procedures—cold exposure, insulin convulsions, electroshock, formalin administered subcutaneously, hypertonic saline intraperitoneally. They suggested that spontaneous hemorrhage occurred only when there was simultaneous interference with several of the mechanisms of hemostasis. To test the validity of the hypothesis further the present series of experiments was designed, in which the influence on hemostasis of the level of circulating platelets singly or in combination with either of the other two factors was studied. Three treatments were administered to induce the desired changes in the hemostatic mechanism. Thrombocytopenia was produced by administering radioactive phosphorus, defective blood coagulation by administering dicumarol, and defective vascular integrity by stress (in the form of 10% NaCl administered intraperitoneally) as in previous experiments (4, 5).

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Materials and Methods

Treatment of animals.—480 female albino rats of the Wistar strain, bred locally, weighing from 80 to 190 g were used over a period of 18 months. P^{32} was obtained from Atomic Energy of Canada Limited, Chalk River, in the form of H_3PO_4 at a concentration of not less than 10 mc/ml. After neutralization it was diluted to a concentration of 400 μc/ml and injected intraperitoneally in a single dose of 4 μc/g of body weight. Dicumarol (Abbott) was mixed with the diet and fed at a dosage of 2 mg/100 g of body weight/day for 7 days. Special feed dishes were used to prevent scattering of feed, and special care was taken to ensure complete ingestion of the prepared food. Ten per cent sodium chloride in a single dose of 2.5 ml/100 g of body weight was injected intraperitoneally using a 25-gauge needle. The control animals were injected intraperitoneally with 0.9% NaCl at the same time as the experimental animals. The environmental conditions were kept as constant as possible. The standard colony diet consisted of Quaker calf meal pellets.

Drawing of blood samples.—The tail was warmed in water at 45 to 50° C, gently massaged and blood samples were drawn from a lateral tail vein using a 25-gauge needle while the animal was wrapped tightly in a towel. The animals were handled daily for 2 weeks before the experiment to become accustomed to the stress of the procedure. Syringes were coated with silicone and needles with Arquad (6). Extreme care was used in obtaining the blood samples. Only blood obtained from clean punctures was used and the presence of air bubbles in the syringe was carefully avoided.

Platelet counts.—Platelet counts under the phase microscope were done according to the technique described by Brecher and Cronkite (7) with one modification: blood was not diluted in the syringe but gently expelled from the 0.5-ml syringe into a siliconed spot dish from where it was aspirated into a red cell pipette.

Prothrombin times.—Prothrombin times were determined on whole blood in a watch glass using a modification of the "bedside method" (8).

General plan of experiments.—Animals were assigned at random to each of the following treatment groups:

(1) P^{32},
(2) dicumarol,
(3) P^{32} and dicumarol,
(4) control,
(5) P^{32},
(6) stress,
(7) P^{32} and stress,
(8) control.

Each experiment lasted 18 days. Platelet counts were taken in all groups on the 1st, 12th, 14th, 16th, and 18th day. Prothrombin times were determined in groups 1, 2, 3, and 4 on the 2nd, 11th, 13th, 15th, and 17th day. P^{32} was administered on the 3rd day to groups 1, 3, 5, and 7. Dicumarol was administered to groups 2 and 3 from the 12th to the 18th day inclusive. Ten per cent NaCl was administered to groups 6 and 7 on the 12th day. At the end of each experimental period, animals were placed in groups on the basis of having platelet counts $<90{,}000/mm^3$ and prothrombin time >60 seconds. These limits were arbitrarily considered to be indicative of marked interference with the two hemostatic factors.

All animals dying during the 18 days of the experiment were subjected to a careful autopsy. Criteria used for classifying an animal as a hemorrhagic death were death; the presence of dark red areas in the internal organs and (or) free blood in the lumen of the intestine and (or) the peritoneal, pleural, and pericardial spaces at the time of post-mortem examination. Occasionally confirmation of the macroscopic findings was obtained by histological examination.

Results and Discussion

The incidence of mortality for different groups of rats is presented in Table I. Of all the theoretically possible combinations of thrombocytopenia and prothrombopenia only those presented in the table were observed.

From the table it can be seen that:

(*a*) in the two groups of rats injected with P^{32}, 70% developed thrombocytopenia, and the mortality was 20% for this latter group;

(*b*) all 50 rats given dicumarol developed prothrombopenia, and the mortality was 16%;

(*c*) in the group of 51 rats given 10% NaCl intraperitoneally the mortality was 4/51; (these were immediate deaths from the hypertonic saline; hemorrhage was not observed and death was distinctly different from that described in *f*);

(*d*) none in the two groups of 50 control rats developed thrombocytopenia or prothrombopenia and the mortality was 0/50 (0%) in each group;

(*e*) 31 of the 49 rats given P^{32} and dicumarol developed thrombocytopenia and prothrombopenia and the mortality was 25/31 (81%);

(*f*) 45 of the 70 rats given P^{32} and stress developed thrombocytopenia and the mortality from hemorrhage was 30/45 (67%).

(*g*) 43 rats which were treated with P^{32} and also received dicumarol or stress did not develop thrombocytopenia. There were no deaths in this group (mortality 0%).

The mortality ratios of the different treatment groups were subjected to the Chi square test. The percentage mortality when thrombocytopenia was combined with either prothrombopenia or stress was significantly greater ($P \ll 0.01$) than the mortality resulting from a single treatment. The percentage mortality from any two treatments combined was significantly greater ($P \ll 0.01$) than that expected from a simple additive effect of the two treatments. The effect of combined treatment in rats with dicumarol and 10% NaCl intraperitoneally was previously reported by Jaques, Mogenson, and Fisher (5) and van Cauwenberge and Jaques (9). The figures reported here agree well with the values previously obtained (8% hemorrhagic death for dicumarol, 9% for immediate deaths from hypertonic saline, and 60% hemorrhagic death from dicumarol and hypertonic saline). Since the time of death, and the ante- and post-mortem findings were the same in the thrombocytopenic rats on combined treatment as had been previously found with animals treated with dicumarol and any one of 10% NaCl administered intraperitoneally, cold exposure, electroshock, formalin given subcutaneously, insulin convulsions, and since these findings are quite different from those resulting from any of these treatments alone (including 10% NaCl given intraperitoneally), we are justified in

TABLE I

Death from spontaneous hemorrhage in rats treated with P^{32}, stress, and dicumarol

Treatment	Platelet count			Prothrombin time			No. rats	No. deaths	% Mortality from hemorrhage
	Thrombocytopenia*	Mean $\times 10^3/mm^3$	s.d.	Prothrombopenia*	Mean, sec	s.d.			
P^{32}	−	505	337	−	34.8	4.3	15	0	0
	+	37	78	−	33.9	7.4	35	7	20
Dicumarol	−	729	155	+	†		50	8	16
P^{32} and dicumarol	−	601	284	+	†		18	0	0
	+	47	93	+	†		31	25	81
Controls	−	739	207	−	33.3	4.4	50	0	0
P^{32}	−	530	383				14	0	0
	+	31	78				40	9	22
Stress	−	747	135				51	4‡	0
P^{32} and stress	−	513	365				25	0	0
	+	39	96				45	30	67
Controls	−	755	276				50	0	0

*Thrombocytopenia refers to a platelet count $<90{,}000/mm^3$; prothrombopenia to a prothrombin time >60 seconds.
†Always >60 seconds: means were not calculated because values often ≫480 seconds.
‡Deaths from immediate effects of the stress procedure, not hemorrhage.

describing the effects observed from hypertonic saline in thrombocytopenic rats as being due to a non-specific stress and, as discussed in previous papers, the effect of non-specific stress is exerted on the vascular component of hemostasis, i.e. the integrity of the blood vessel wall.

A rather remarkable incidental observation was that no deaths at all occurred in spite of the administration of dicumarol or stress in those P^{32}-treated animals which did not develop thrombocytopenia. Since the expected incidence would be low (8%), the number of animals is too small to put too much reliance on the observation. However, this has been observed in experiments of this type with other treatments and may be due to some interaction such as the P^{32}-injections being a mild stress interfering with later effects, as observed by Mogenson and Jaques (10).

Hemostasis in mammals is maintained by three interrelated mechanisms: platelet massing, formation of fibrin, and integrity of the blood vessel wall. The results of the experiments reported here again show that interference with any one of these three mechanisms fails to produce death from spontaneous hemorrhage but, when more than one mechanism is interfered with, the incidence of death from hemorrhage becomes much higher than the expected combined mortality. These results provide additional support for the hypothesis that three closely interrelated mechanisms take part in the maintenance of hemostasis and several of these must be deranged for spontaneous bleeding to occur. Further, these results provide direct evidence that an adequate number of intact platelets is required for one of the mechanisms.

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THE EFFECT OF BOUND INSULIN ON THE RESPIRATORY RATE AND ELECTROLYTES OF INTACT FROG MUSCLE[1]

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Abstract

Isolated frog muscles were immersed in Ringer's solution containing insulin, for varying periods of time from 5 to 40 minutes; following this procedure they were washed, with varying degrees of intensity, in insulin-free Ringer's solution. Contralateral muscles were treated similarly with insulin-free Ringer's solution. The muscles then respired in Warburg vessels in the presence of lactate for 6 hours, at which time the media in the Warburg vessels were analyzed for potassium and phosphate and the muscles for water and sodium. Data from paired muscles were always compared. Results from several experimental designs led to the conclusion that insulin was held in some way to the muscle and was carried over into the Warburg vessel since marked stimulation of respiratory rate, potassium and phosphate uptake, and sodium loss were observed. Extensive washing did not remove the bound insulin. No greater effect of insulin was observed when it was bound at a pH of 7.6 than at a pH of 6.0.

Introduction

The literature pertaining to insulin binding was reviewed in detail by Stadie in 1954 (2) and by Williams in 1956 (3). In brief, the following observations have been presented in support of the concept that insulin can combine with some component of tissues. (*a*) When rat diaphragm was soaked momentarily in an insulin solution, washed, and then allowed to respire in a glucose medium, it synthesized glycogen at a more rapid rate than controls not previously soaked in insulin (4). (*b*) The combination of insulin with the diaphragm occurred under nitrogen, was temperature-dependent, and was impaired by the anterior pituitary hormone (4, 5). (*c*) Using isotopically labelled insulin the effect on glycogen synthesis was proportional to the insulin bound (6). (*d*) Insulin was bound by a wide variety of tissues (liver, kidney, and to a lesser extent by muscular, cardiac, and skeletal tissue (7; see also Rose and Nelson (8)) and by human erythrocytes, rat and rabbit leucocytes, thymus and bone marrow cells, mouse sperm, and yeast cells (9).

Of even more interest than the combination of insulin with the intact cell is its binding to the following tissue components: (*a*) red cell fragments (9), (*b*) liver and kidney cellular fractions in order of decreasing amounts: residual > mitochondrial > nuclear > microsomal (10); (*c*) reticulin fibers of rat spleen and diaphragm (11). (*d*) Finally, the physical binding of insulin by gamma globulins appears to be established (12, 13). Although no one would deny that some sort of combination is a prerequisite for a chemical reaction between substances to take place, the nonspecificity of insulin binding detracts somewhat from the physiological significance of this phenomenon. Newerly and Berson (14) believe

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[2]Postdoctoral Medical Research Fellow of the National Research Council of Canada.

this is an example of nonspecific surface adsorption of proteins since labelled serum albumin, globulin, and glycogen as well as insulin bind to diaphragms (either fresh or formaldehyde treated) and are eluted slowly on continuous washing. Reference was also made to insulin adsorption on paper and glass.

On the other hand, Stadie pointed out (15) that the amount bound to tissues with high rates of metabolism was high. It was anticipated that active skeletal muscle might exhibit binding to a marked degree. Muscles of the hind leg of the frog had previously been shown to respond to insulin by an increase in respiratory rate and by the uptake of potassium with or without lactate being present (16, 17, 18, 19). These effects of insulin were used as tools in the present research to demonstrate that insulin could combine tightly with frog muscle (1). Almost simultaneously and quite independently Gourley (20) carried out a series of experiments also designed to establish this fact.

In this report, data showing insulin-induced stimulation of respiratory rate and potassium and phosphate uptake after short preliminary exposure of frog muscle to insulin are interpreted as evidence of a firm binding by frog muscle. The insulin could not be removed by much more excessive washing than that used by Gourley and considerably higher amounts of potassium were taken up than those which he reported. Also it was shown that the same amount of insulin was bound at pH 6.0 as at pH 7.6.

Materials and Methods

Common 'leopard' frogs (*Rana pipiens* Schreber) were used for all the experiments. They were collected locally and were kept in cement tanks with a continuously running water supply, the temperature of which varied from 4 to 14° C throughout the year. The frogs which supplied the data for the first series of experiments were fed one dew worm a week throughout spring and summer; those used in the second and third series were fed at the same rate throughout the entire year while those for the fourth series were unfed. Animals were discarded which showed any abnormality: encysted worms, bone fractures, 'red leg', or any hemorrhagic condition involving skin, connective tissue, or muscle.

As in previous researches (17, 18, 19) four muscles were carefully dissected from the posterior limbs, weighed, and soaked in Ringer's solution at 4 to 5° C overnight. The Ringer's solutions, which were buffered with phosphate and contained calcium but no magnesium, were essentially of the same composition for each series but differed in the concentrations of potassium, which was 6 meq per liter for the experiments of series 1, 2, and 3 and 2 meq per liter for those of series 4. The higher concentration of potassium was used in the first two series because, when potassium movement was being investigated by Smillie and Manery (19), it was found that the insulin-induced potassium uptake was greater at 6 meq K per liter than at the lower potassium concentration. The sartorius and tibialus anticus longus muscles were used together as one set (M_1) and the semitendinosus and ileofibularis as the second set (M_2). In all experiments a set of muscles from one leg served as the experimental tissue and the corresponding set from the opposite leg as the control.

The pertinent details of each experimental design will be given in each section of the results. In all cases the muscles respired in pure oxygen in Warburg respirometers at $20 \pm 0.05°$ C and the oxygen consumption was measured for 6 hours. At the end of the respiratory period the muscles were shown to be contractile, were weighed, and, when sodium and potassium analyses were to be done, were placed in tared platinum crucibles for dry weight determinations (100° C) and ashed overnight in a muffle furnace at 550° C. Potassium determinations were carried out on a sample of the fluid from each Warburg vessel (19) using a flame photometer (Perkin-Elmer, 52-A), with the precautions and accuracy described by Barlow and Manery (21).

Results

Series 1. Bound Insulin and Added Insulin (Each in Ringer–Lactate) versus Ringer's Solution Alone

In this series of experiments one set of muscles (e.g. M_1 right) from each frog, after being in cold Ringer's overnight, was placed for 5 minutes at 20° C in Ringer's solution containing 0.5 unit of insulin* per ml. The muscles were then rinsed in 15 ml of insulin-free Ringer's solution and were washed twice for 10 minutes in 10-ml samples at 0° C in order to permit the insulin to diffuse out of the tissue spaces into the external medium. If the extracellular fluid volume is 20% of the weight of these muscles, and if insulin should diffuse freely into the medium, the insulin concentration in the tissue spaces will be reduced by this procedure to about 8×10^{-6} unit per ml, a concentration too low to produce any effect (17). The tissue was blotted gently between each fluid change to remove excess adhering fluid. The contralateral muscles (M_1 left) were soaked, washed, and in all details treated identically except for the lack of insulin in the fluid in which they were soaked for 5 minutes. All muscles were then placed in Ringer's solution in Warburg vessels and the rates of oxygen consumption observed for the first 2 hours. The muscle set pretreated with insulin is called the experimental muscle; the untreated set from the opposite leg serves as the control.

At the end of the second hour sodium lactate was added to the experimental muscles from the sidearm of the Warburg vessels, and an equivalent amount of Ringer's solution added to the control muscles so that the latter respired in Ringer's solution throughout. Respiration was measured for the next 3 or 4 hours. Any differences between the experimental and the control muscles are caused by the lactate and the insulin bound to the experimental muscle.

A second pair of muscle sets (e.g. M_2R and M_2L) was taken from each frog to demonstrate the effect of added insulin + lactate; these were not subjected to any preliminary treatment. Table IA illustrates the experimental design. Both the experimental and control sets respired in Ringer's solution for the first 2 hours, at which time a mixture of Ringer's + insulin + lactate (RIL) was added to the experimental muscles and Ringer's solution only to the controls. Thus in each frog the effect of bound insulin + lactate was demonstrated

*Crystalline zinc insulin was obtained through the courtesy of Dr. D. A. Scott, of the Connaught Laboratories, University of Toronto.

TABLE I

The effect of lactate and bound insulin or lactate and added insulin on the respiration and K movement in the muscles of late summer frogs (Aug., Sept., Oct.) (Insulin conc. for binding, 0.5 unit/ml; final insulin conc. when added, 0.05 unit/ml; final lactate conc., 0.005 *M*; initial pH of Warburg solutions, 7.4)

A. Experimental design

Control (C)		Experimental (E)		Warburg solutions			
				Main space		Sidearm	
Musc. set	Treatment	Musc. set	Treatment	C	E	C	E
M_1R	Soaked in R, washed	M_1L (IB)	Soaked in RI, washed	R	R	R	RL
M_2L	—	M_2R (IA)	—	R	R	R	RIL

B. Experimental data

Exptl. musc.	Rate of O_2 consumption (cu.mm/g/hr)						Total O_2 (cu.mm/g/6 hr)		Musc. K. change (meq/kg)		
	2nd hr		Final		ΔO_2E (5) − (3)	IL effect $\Delta O_2E - \Delta O_2C$					IL effect ΔKE − ΔKC
	C	E	C	E			C	E	ΔKC	ΔKE	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
IB	33.4	35.4	40.4	66.5	+31.3	+24.6	199.9	283.1	−2.02	+2.91	+4.93
	±2.2	±2.8	±3.9	±5.0	±5.1	±2.3	±9.9	±10.6	±0.59	±0.63	±0.63
IA	35.9	35.2	36.9	65.6	+29.0	+27.1	196.1	278.6	−1.69	+4.42	+6.11
	±2.0	±2.9	±3.0	±4.4	±4.9	±3.6	±7.8	±10.2	±0.97	±0.88	±1.03

NOTE: (*a*) Each of nine frogs supplied four muscle sets, an insulin-bound muscle (IB = E) and its control (C) and an insulin-added muscle (IA = E) and its control; these were randomized so that no bias was introduced by anatomically different muscles or by left and right sidedness.

(*b*) Each figure is the true mean ± the standard error of the mean calculated for nine muscles or nine pairs (col. 7 and 12). Data are expressed in units per g or kg final wet weight.

(*c*) Meaning of symbols: M_1R and M_1L = muscle set 1, right and left; IB = insulin bound; IA = insulin added; R = Ringer's solution; RL = Ringer's + lactate; RI = Ringer's + insulin; RIL = Ringer's + insulin + lactate; IL = insulin + lactate. ΔO_2E and ΔO_2C = difference between second hour rate and final rate of O_2 consumption for experimental and control muscles, respectively. ΔKE and ΔKC = uptake of K by experimental and control muscles, respectively, calculated from analyses of media in Warburg vessels at the end of the experiment.

in addition to the effect of added insulin + lactate.

In Table IB the data from 36 muscle sets (nine frogs) are presented. In spite of the fact that the experimental muscles IB were pretreated with insulin, they respired during the first 2 hours at the same rate as the controls which were not exposed to insulin (col. 2 and col. 3). Hence the insulin which was carried into the Warburg vessel by the muscles had no observable effect on the initial respiration. Addition of lactate to these muscles with bound insulin, however, accelerated the *rate of oxygen utilization* on the average by 100% (range 42% to 230%), from a mean value of 35.4 (col. 3) to 66.5 cu. mm per g per hour (col. 5). The difference between the second hour rate and that at the fifth and sixth hours was +31.3, a value designated as ΔO_2E in accord with previous publications (18, 19); the respiratory rate of the control muscles changed very little being 33.4 (col. 2) at the end of the second hour and 40.4 (col. 4) at the termination of the experiment. The net stimulation by bound insulin + lactate (i.e. $\Delta O_2E - \Delta O_2C$) amounted to +24.6 cu. mm per g per hour (col. 7).

Although the second group of muscles (IA) experienced much less handling than those in group 1, the second hour rates (col. 2 and 3) were remarkably similar to those of group 1. The control muscles continued at much the same rate during the entire experiment (col. 2 and 4) while the addition of insulin + lactate at the end of the second hour greatly stimulated the rate (col. 5). It may be fortuitous that the average final rate of 65.6 for muscles to which insulin and lactate has been added is identical with that of 66.5 found for muscles previously exposed to insulin (lactate added) and that the insulin–lactate effects (col. 7) are about the same (+24.6 and +27.1).

The *total oxygen* consumed is the same for all control muscles being about 200 cu. mm/g/6 hours (col. 8). In the presence of lactate the total oxygen consumed was the same (about 280 cu. mm/g/6 hours, col. 9) whether the insulin had been bound during a pre-experimental treatment or added at the end of the second hour.

Control muscles respiring in Ringer's solution even with 6 meq potassium per liter have been previously observed to lose a small amount of *potassium* (18, 19). This loss is evident from the figures in col. 10, Table IB. Not only is this loss abolished but potassium is taken up by the muscle from the medium, when the muscle is exposed to lactate either with bound or added insulin (col. 11). The net effect of the insulin + lactate in both groups is highly significant being an uptake of +4.93 for group 1 muscles and +6.11 for group 2 muscles.

Statistical procedures ('*t*' test for non-paired variates) were applied to the effect of lactate + bound insulin and of lactate + added insulin on the rate of oxygen consumption (col. 7) and on the potassium change (col. 12). It was found that the insulin–lactate effect on oxygen consumption of +24.6 was not significantly different from +27.1 at the 0.05 level, and that the potassium uptakes of +4.93 and +6.11 were statistically the same. Apparently similar responses were elicited in the presence of lactate whether insulin was present in the medium throughout the experiment or was applied to the muscles only for a short period prior to the Warburg experiment.

The question arises as to whether or not this effect is due to lactate, to insulin, or to the combination of insulin and lactate since both were added to the experimental muscles and not to the controls. Lactate (0.005 *M*) had previously been shown to stimulate the rate of oxygen consumption of this muscle preparation in the absence of insulin; in the presence of insulin there was a further stimulation of the respiratory rate (17, 18, 19). For this reason it can be assumed that these data of Table IB, col. 7, show a combined insulin + lactate stimulation. This conviction is strengthened by the fact that lactate alone produced little if any uptake of potassium whereas insulin + lactate and insulin alone caused increases of 6 to 8 meq per kg of muscle (18, 19), which is of the same order of magnitude as the changes reported here (Table IB, col. 12). In order to demonstrate the influence of insulin in an unequivocal fashion experiments of series 2 were performed.

All muscles in series 1 were analyzed for *glycogen* at the termination of the Warburg experiment. In eight pairs out of nine the muscles pretreated with insulin had more glycogen than their contralateral controls, the average values being 9.44 ± 0.93 (mg glucose/g final wet wt.) for insulin-bound muscles and 8.63 ± 0.92 for their paired controls. This difference might be attributed to the effect of lactate. No such consistent differences, however, were found between the muscles to which insulin had been added in the Warburg vessels and their nine paired controls; the average values were 8.79 ± 0.93 and 8.79 ± 0.75 respectively. Both types of muscles were exposed to lactate for the same length of time. Hence, although the series is small for a glycogen study, the data suggest that during the longer period of time to which the pretreated muscles were exposed to insulin without added lactate some changes occurred which resulted in a net increase in glycogen over the amount in the controls.

Series 2. Bound Insulin and Added Insulin (Each in Ringer–Lactate) versus Ringer–Lactate

In the above series of experiments the insulin effect could not be separated from the lactate effect, since at the end of the second hour Ringer's solution was added to all control muscles and Ringer's + lactate to all experimental muscles (Table IA). Although it has been repeatedly observed that the stimulation by insulin and lactate are additive, nevertheless it was felt necessary to observe a true effect of insulin separated from the influence of lactate.

Accordingly, the experimental design shown in Table IIA was adopted along with the same soaking, rinsing, and washing procedures as those used in series 1. In the experimental vessels insulin was present throughout, since it was bound in the IB muscles and was added at the outset to the main space of the Warburg vessels which would contain the IA muscles. Lactate was added to both the controls and the experimentals at the end of the second hour. The differences between the paired muscle sets must be attributed to either insulin bound or insulin added. This series of experiments differs from series 1 in another respect, i.e. that the muscles to which insulin was added in the Warburg vessels were soaked and washed in insulin-free Ringer's and, in every detail were exposed to the same manipulations as those previously exposed to insulin (see Table IIA).

TABLE II

The effect of insulin (bound or added) on the respiration and K movement in muscles of October frogs
(All muscles were exposed to lactate; concentrations and symbol meanings are listed in Table I)

A. Experimental design

Control (C)		Experimental (E)		Warburg solutions			
				Main space		Sidearm	
Musc. set	Treatment	Musc. set	Treatment	C	E	C	E
M_1R	Soaked in R, washed	M_1L (IB)	Soaked in RI, washed	R	R	RL	RL
M_2L	Soaked in R, washed	M_2R (IA)	Soaked in R, washed	R	RI	RL	RL

B. Experimental data

Exptl. musc. (E)	Rate of O_2 consumption (cu.mm/g/hr)						Total O_2 (cu.mm/g/6 hr)		Musc. K changes (meq/kg)		
	2nd hr		Final		ΔO_2E	I effect					
	C	E	C	E	(5)−(3)	$\Delta O_2E-\Delta O_2C$	C	E	ΔKC	ΔKE	I effect ΔKE−ΔKC
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
IB	36.8	37.3	61.3	74.4	+37.1	+12.3	305.5	345.6	−4.02	+3.30	+7.32
	±3.1	±2.2	±4.8	±4.8	±4.3	± 2.9	±22.5	±22.2	±0.82	±0.71	±0.91
IA	34.8	37.4	64.2	80.9	+43.3	+14.0	312.1	375.6	−3.92	+9.22	+13.14
	±2.9	±3.0	±4.9	±4.4	± 2.8	± 4.9	±19.5	±19.6	±0.85	±0.63	±1.21

NOTE: Each figure is the mean (± standard error of the mean) of values from six muscles or six muscle pairs.

The rate of respiration in the first 2 hours in Ringer's solution alone (Table IIB, col. 2) was almost identical with that in Ringer's (col. 3) to which insulin had been added (IA) or in which insulin was bound to the muscle (IB). A stimulation of about 75% due to lactate alone was observed in both control series (col. 4), and the rate was still further accelerated when insulin was present, reaching a final rate of 74.4 cu. mm/g/hour, when the muscle had been previously exposed to insulin (IB) and 80.9 when insulin was added (IA). The stimulation due to insulin (col. 7) was the same whether the insulin had been carried over into the Warburg vessel as a result of previous exposure or added initially to the main space of the Warburg vessel, the values being +12.3 for the IB group and +14.0 for the IA group. There is also an insulin stimulation observed in the *total oxygen* consumed over the entire 6-hour period (col. 9 minus col. 8).

As in series 1, all control muscles lost *potassium* (col. 10) while the experimentals gained potassium (col. 11). The insulin effects (col. 12) of both bound and added insulin were greater than in series 1. This finding cannot be attributed to the difference in experimental design but to the season. In this series there appeared to be a greater insulin-induced potassium uptake when the hormone was added than when it was combined with the muscle during exposure prior to the respiratory experiment. The conclusion is inescapable that insulin was carried over into the Warburg vessel tightly attached in some way to the muscle.

Series 3. pH and Insulin Binding

If insulin must become closely associated or combined with certain groups on the surface of cells before it can exert its effect, it is conceivable that it might do so by simple electrostatic bonding. Since the isoelectric point of insulin is 5.33 (22), the molecule would have a net negative charge at pH 7.4, the pH of frog plasma; the net negative charge would decrease as the medium became more acid. Less insulin might be expected to combine with muscle components electrostatically at pH 6.0 than at pH 7.6. This series of experiments was designed to compare the effects of insulin bound in alkaline media (pH 7.6) and in acid media (pH 6.0) with the effects of added insulin. The experimental design is shown in Table IIIA. Each frog provided a pair of muscle sets for alkaline treatment and a pair for acid treatment. One set of the alkaline-treated pair bound insulin and was compared to its contralateral set to which insulin was added. Similarly, one set of the acid-treated pair bound insulin and was compared to its contralateral set to which insulin was added.

The details of the procedure follow. The muscles were soaked overnight in Ringer's solution. Then one set was soaked for 5 minutes at 20° C in Ringer's solution at pH 7.63 containing 0.5 unit insulin per ml. The contralateral set was soaked in Ringer's at pH of 7.59 (essentially the same as 7.63). These were the alkaline-treated muscle sets. Similarly one set of the acid-treated pair was soaked for 5 minutes at 20° C in Ringer's solution at pH 6.01 containing 0.5 unit of insulin per ml; the contralateral set was similarly soaked in Ringer's solution at a pH of 5.99. Following the initial brief soaking period to permit the muscles

TABLE III

The effect of insulin bound in alkaline (pH 7.61) and acid (pH 6.0) Ringer's on respiratory rate and electrolytes of muscles of December frogs
(All muscles were exposed to lactate; concentrations and symbols are listed in Table I)

A. Experimental design

Insulin added		Insulin bound		Warburg solutions pH 7.46			
				Main space		Sidearm	
Musc. set	Treatment	Musc. set	Treatment	IA	IB	IA	IB
M_1R	Soaked in R, pH 7.59, washed	M_1L	Soaked in RI, pH 7.63, washed	RI	R	RL	RL
M_2L	Soaked in R, pH 5.99, washed	M_2R	Soaked in RI, pH 6.01, washed	RI	R	RL	RL

B. Experimental data

pH for insulin binding	Rate of O_2 consumption (cu.mm/g/hr)						Muscle changes					
							ΔK		ΔPi		Na conc.	
	2nd hr		Final		I effect		IA	IB	IA	IB	IA	IB
	IA	IB	IA	IB	IA	IB	mmoles/kg final wet wt.					
(1)	(2)	(3)	(4)	(5)	(6) (4)−(2)	(7) (5)−(3)	(8)	(9)	(10)	(11)	(12)	(13)
7.59−7.63	34.4	33.2	59.3	55.5	+24.9	+22.3	+7.9	+4.5	+3.8	+3.5	36.9	39.7
5.99−6.01	37.4	33.0	59.1	54.7	+21.7	+21.7	+7.6	+4.7	+3.8	+3.5	39.5	43.9

NOTE: Each figure represents the values from three muscle sets of three muscle pairs. Each frog provides a pair of muscle sets for acid soaking and a pair for alkaline soaking; to one member of a pair insulin is added (IA); to the other insulin is bound (IB).

to bind insulin at different levels of pH, all sets were blotted dry, rinsed quickly, and soaked for two successive periods of 10 minutes at 0° C in 10 ml Ringer's solution at pH 7.45 to remove any excess insulin from those exposed to it. These sets (IB sets) were loaded in Warburg vessels (chilled) containing Ringer's solution (pH 7.45) in the main space, while their contralateral muscle sets (IA sets) were placed in Warburg vessels in Ringer's containing 0.05 unit of insulin per ml at essentially the same pH (7.47). After 2 hours Ringer's + lactate was added to all sets from the sidearms and the oxygen consumption rates were measured for a further 4 hours. Then appropriate fluid analyses were made for potassium, phosphate, and pH. The muscles were dried, ashed, and analyzed for sodium. Examination of data from the alkaline-treated muscle sets should reveal whether or not sufficient insulin was bound in 5 minutes at pH 7.6 to give the same muscle response as the contralateral sets which were exposed to added insulin for the entire 6-hour incubation period. A similar comparison could be made between contralaterally paired sets which were acid-treated. Finally, since each frog provided both alkaline- and acid-treated sets, a comparison should indicate the relative amounts of insulin bound at the two pH values.

Table IIIB shows that there were no differences in the *rates of oxygen consumption* of the four groups of muscle sets during the second hour (col. 2 and 3). The average respiratory rates for the fifth and sixth hours of the insulin bound (IB) sets were the same at both pH values, being 55.5 and 54.7 cu. mm/g/hour. Although these appear to be slightly less than the analogous rates of 59.3 and 59.1 for the contralateral sets to which insulin had been added (IA), the insulin effect shown in columns 6 and 7 are the same whether the insulin was bound or added. The *total oxygen* data also showed that there was no difference in the amount of insulin bound in 5 minutes at 20° C at a pH of 7.6 and at a pH of 6.0.

Analogous effects on potassium, sodium, and phosphate data were observed. The average *potassium* uptake by IB muscles (col. 9) was independent of the pH of the solution in which the muscles were previously exposed to insulin. But in each pair the uptake (col. 8) was greater in the IA contralateral sets which were exposed to insulin throughout the 6-hour experimental period than in the IB sets. Thus the added insulin appeared to induce a greater potassium movement than did bound insulin, although the two methods of administering the hormone stimulated the respiratory rate to the same degree. This is another example, of which we now have several (18, 19), of the independence of potassium uptake and oxygen consumption.

Inorganic phosphate had previously been shown (23) to enter frog muscles under the influence of insulin and lactate. The data of columns 10 and 11 demonstrate that bound insulin has the same influence as added insulin on the uptake of phosphate under the conditions of these experiments. Moreover, no difference was observed between the alkaline-treated and acid-treated muscle sets.

Similarly, the *sodium* concentrations of the muscles at the end of the experiment did not differ appreciably under the four conditions studied. It has also

been established, although as yet unpublished, that under the influence of insulin and lactate frog muscles lose some of the sodium which they imbibe during overnight soaking. From the average concentrations known to be present in such muscles at the beginning of the Warburg experiment the muscles described in Table IIIB appear to have lost similar amounts of sodium whether the insulin is bound or added.

It should be pointed out that the concentration of *water* (80.9–81.6% wet weight) did not vary appreciably among the four groups of muscles. Furthermore, the pH of the media in the Warburg vessels rose from 7.47 to a *final average pH* of 7.66 and 7.60, for the alkaline-treated insulin-bound muscles and their paired insulin-added sets respectively, and to 7.52 and 7.57 for the acid-treated insulin-bound muscles and their paired insulin-added sets, respectively. It is of some interest to note that the final pH of the media containing muscles previously exposed for only 5 minutes to a slightly alkaline medium, was higher than that surrounding muscles similarly exposed to a more acid medium. It has been established (24) that insulin induces potassium and phosphate uptake in media at both low and high pH values.

The above results indicate that there is no detectable difference in the binding of insulin during 5 minutes' exposure to Ringer's solution at pH 6.0 and 7.6. The pH range is, of course, small and other factors such as ionic strength, size of the insulin aggregate, speed of diffusion to the binding sites, time of exposure, etc., may all influence the amount of insulin combined with the muscle.

Series 4. Diffusion of Insulin

The pattern of soaking in insulin and of rinsing and washing was the same in the experiments of series 1, 2, and 3. It may have been fortuitous that a 5-minute exposure to 0.5 unit of insulin per ml, after exhaustive washing, produced effects almost identical with those observed when 0.05 unit of insulin per ml was present throughout the 6-hour experimental period. But there is an implication from the similarity of the effects that this design produced maximal binding.

In the experiments of series 4, performed by one of us (J.C.D.) different times of exposure to insulin (0.033 unit/ml) and varying rinsing periods were used, and the data again provide strong support for the view that insulin was in some way tightly held to frog muscle during the preliminary soaking treatment.

In these experiments one member of a pair of muscle sets, prepared as in previous experiments, was put into Ringer's solution containing 0.033 unit of insulin per ml. The other member was placed in insulin-free Ringer's solution. After 10, 20, 30, or 40 minutes both sets were removed, blotted on filter paper, rinsed for 1 minute in a large volume of Ringer's solution in order to remove the insulin on the outside of the experimental muscles, blotted again, and put into Warburg vessels containing a final concentration of 0.01 *M* lactate.

Undoubtedly after the relatively long soaking periods and only 1 minute of rinsing, insulin would remain in the tissue spaces. Since the rates of O_2 consumption were calculated from the last 3 hours of a 4½-hour period, this would leave 1½ hours for the insulin in the tissue spaces to diffuse into the external

medium (1.6 ml) and the insulin concentration would be too low to produce a measurable effect (17). The rates of O_2 consumption for 12 pairs are shown in Table IVA where a definite stimulation by insulin can be seen for each period of

TABLE IV

Rate of O_2 consumption (cu.mm/g/hr) after varying the period of exposure to insulin (One member of a pair of muscle sets was soaked in Ringer's + insulin (0.033 unit/ml) for the times indicated; the other member was similarly treated with insulin-free Ringer's solution. All muscles respired in 0.01 *M* lactate (see text). Each figure in A is the average of three muscle sets)

A

	Minutes in insulin							
	0	10	0	20	0	30	0	40
O_2 consumption rate	53.6	65.7	55.5	71.5	46.9	63.2	52.6	73.6
O_2 increase (cu.mm)		+12.1		+16.0		+16.3		+21.0
O_2 increase (%)*		+22.6		+28.6		+34.8		+39.9

B

Muscles and treatment	O_2 rates in insulin 0 min	25 min	O_2 increase (cu.mm)	O_2 increase* (%)
12 pairs, average of A rates after insulin, 1-min washing	52.5	68.5	+16.3†	+31.5
8 pairs, 25-min washing in five solutions	78.7	95.7	+17.0†	+21.6
Average	62.8	79.3	+16.5	+26.3

* Increases in rate of O_2 consumption due to insulin are expressed as percentages of the rates of the contralateral sets not exposed to insulin.
†According to "*t*" test there is no significant difference between the increases in rate due to insulin.

immersion in Ringer–insulin solution. The longer a muscle is exposed to insulin, the greater is the absolute increase in rate of O_2 consumption. Moreover, the percentage increase due to insulin (i.e. increase expressed as a percentage of the rate in muscles not exposed to insulin) becomes greater with increased exposure. The results also suggest that the increase caused by pre-exposure to insulin for 30 to 40 minutes is reaching a plateau level, as though maximal binding were almost attained.

Since insulin diffuses easily into the muscle, it might be supposed that much of it would diffuse out with extensive washing in insulin-free Ringer's solution. The preceding experiments showed that enough insulin to cause an effect entered the muscle in exposures of 10, 20, 30, and 40 minutes, and that this amount will not diffuse out in times up to $1\frac{1}{2}$ hours. To test further the reality of the binding of the insulin thus implied, another series of experiments was performed, identical with that just described, but using a 25-minute period of immersion in insulin, followed by a 25-minute washing period in which the solution was completely renewed every 5 minutes. The rates were determined as before, and it may be seen in Table IVB (col. 4) that the average absolute increases in respiratory rates were identical (+16.3 and +17.0). It is to be noted that the control muscles (col. 2), which were handled so frequently by many washings,

respired at a much higher rate (78.7 cu. mm) than those which were only washed once (52.5 cu. mm). As a consequence the insulin effect, when expressed as a percentage of the control rate (col. 5) was considerably lower in extensively washed muscles (+21.6%) than in those receiving much less handling (+31.5%). Accepting as correct the judgement of the insulin effect from absolute rather than percentage increases in rate, it is necessary to conclude that no insulin was washed out of the muscles by repeatedly washing them. It seems evident that insulin enters much faster than it escapes.

Discussion

In the first three series of experiments 5 minutes at 20° C was sufficient for insulin (0.5 unit/ml) to diffuse into the muscle to a site at which it could exert biological activity. Here enough of it remained to cause stimulation, even after one brief rinsing at 20° C and two washing periods of 10 minutes each, at 0° C. Although not directly measured, the presence of insulin in the muscle seems assured, because of the finding by others (6, 7, 14) that the isotopic label remained in tissues for long periods after they were exposed to isotopically labelled insulin. Newerly and Berson (14) found an appreciable portion still present in isolated diaphragm after 1½ hours in insulin-free solutions which were frequently renewed. Their data showed a significant decrease to a plateau level in this time and a slight but progressive diminution thereafter. There is no indication in our data of an appreciable diminution in the effect of insulin during a 6-hour experiment following intensive washing. Hence diffusion from the muscle, if it occurred, must have been too slow to be observed. In series 4, also, when 0.033 unit of insulin per ml was used for binding followed by extensive washing procedures, the effect of insulin was just as great as after one short rinsing period. It is difficult to escape the conviction that insulin has become bound to the muscle during the exposure period.

Although reference has been made to bound insulin throughout this presentation there is no implication of a knowledge of mechanism. Insulin is a large molecule and can be expected to diffuse slowly but it enters muscles much faster than it escapes. The functional "barrier" which is apparently imposed on the exit is referred to as "binding".

Only brief exposure periods are required to produce large subsequent effects and it is inconceivable that these effects result from the stimulation of surface fibers only. On the other hand, Bleehen and Fisher (25) appear to have reduced markedly, in 30 minutes, the so-called bound insulin from a perfused rat heart preparation and to have removed the insulin almost completely in 60 minutes. Whether the perfusion technique provides more adequate diffusion, or heart muscle does not contain the types of structures necessary to hold insulin firmly must remain undecided at this time. Rose and Nelson (8) found perfusion of the whole animal removed only a small part of the radio-iodinated insulin from liver, kidney, and skeletal muscle.

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THE BIOCHEMISTRY OF THE NITRIFYING ORGANISMS

PART 7. THE PHOSPHATE COMPOUNDS OF NITROBACTER AND THE UPTAKE OF ORTHOPHOSPHATE BY THE ORGANISM[1]

W. D. BUTT[2] AND H. LEES[3]

Abstract

The phosphate compounds present in the cells of the chemosynthetic autotrophic bacterium *Nitrobacter* were fractionated by means of successive extractions with cold 5% trichloracetic acid, ethanol, ethanol:ether, and dilute alkali. It was found that about 50% of the total cell phosphate was soluble in dilute alkali and, of this, 76% was acid-labile. Treatment of the alkali extract with Norit reduced the content of non-labile phosphate without affecting the acid-labile component; this labile compound was identified as a polyphosphate. By the use of ^{32}P-labelled orthophosphate, it was shown that the cells absorbed orthophosphate from the medium at a slow rate during nitrite oxidation.

Introduction

Little attention has been devoted to the phosphorus metabolism of *Nitrobacter*. Simpson (1) found that the organism grew in a medium devoid of any phosphate save that added with the inoculum; even when thick suspensions of cells oxidized nitrite, there was only a very small disappearance of inorganic phosphate from the incubation medium. We have now shown that inorganic phosphate is utilized by *Nitrobacter* and have made a preliminary examination of the intracellular phosphorus compounds.

Methods

Nitrobacter cells were grown and harvested by methods already described (2).

Fractionation of Phosphate Compounds

The method used was essentially that of Mudd, Yoshida, and Koike (3). Washed cells (about 25 mg nitrogen) were suspended in 12 ml of ice-cold 5% trichloracetic acid (T.C.A.) and 2 ml taken for estimation of total nitrogen and total phosphate. After extraction for 15 minutes at 0°, the cells were centrifuged at 0° and washed twice by centrifuging with 2.5-ml portions of ice-cold T.C.A. The extract and washings were pooled, made to 15 ml, and the orthophosphate, acid-labile phosphate, and total phosphate determined.

The residue from the T.C.A. extraction was extracted with 5 ml of 70% v/v aqueous ethanol for 18 hours at room temperature and then with 6 ml of ethanol:ether (3:1) at 40–50° for 4 hours. After these extractions, the residue was washed twice by centrifuging with 2 ml of ethanol:ether and the pooled extracts and washings made to 15 ml with ethanol for total phosphate estimation.

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The residue from the ethanol:ether extraction was suspended in 10 ml water, brought to pH 8–9 with 0.1 *N* NaOH, and extracted for 3 hours at room temperature. After a further extraction with 3 ml of alkali at the same pH for 1 hour at room temperature, the residue was washed once by centrifuging with 2 ml of alkali at the same pH. The extracts and washing were pooled and made to 15 ml for orthophosphate, acid-labile phosphate, and total phosphate estimations.

The final residue was suspended to 5 ml in water and total phosphate estimated.

Estimation of Orthophosphate

Orthophosphate was determined by the method of Fiske and Subbarow (4). The color was measured in an E.E.L. colorimeter (Evans Electroselenium Ltd., Harlow, Essex, England) with a red filter O.R.1 (maximum transmission at 680 mμ).

Estimation of Acid-labile Phosphate

This was measured as the orthophosphate liberated on hydrolysis for 7 minutes at 100° with 1 *N* HCl.

Estimation of Total Phosphate

The total phosphate content of samples was estimated by the method described by LePage (4). Most of the samples were fully hydrolyzed after digestion in concentrated H_2SO_4 for about 60 minutes but a digestion period of about 120 minutes was found necessary for the ethanol:ether-soluble fraction.

Detection of Phosphate on Paper Chromatograms

Phosphate was detected by spraying with the ammonium molybdate spray of Hanes and Isherwood (5). After spraying, the papers were dried, heated at 85° for 7 minutes, and treated with moist H_2S to develop the blue spots.

Measurement of ^{32}P in Extracts

A suitable volume of extract was absorbed on 15 disks of lens tissue fitted in the well of a polythene planchette having a well area of 1 sq. cm. The radioactivity was then measured immediately, without prior drying of the tissue by means of a thin mica end-window Geiger tube (20th Century Electronics Ltd., New Addington, Surrey, England). Counts were registered over a period of 1000 seconds by an Ekco N530D automatic decatron scaler (E. K. Cole Ltd., Southend, Essex, England). Correction for background was made. To estimate the specific activity of the phosphate supplied to the cells, the incubation medium was treated with barium acetate at 0° and pH 8.2. The precipitate of barium phosphate obtained was washed with water, dissolved in 0.05 *N* H_2SO_4, and the precipitate of barium sulphate centrifuged off. The radioactivity of the supernatant solution and its orthophosphate content were determined in the usual ways. No correction for the decay of ^{32}P was made in any experiment since all samples from one experiment, including samples for specific activity determinations, were counted within a short time of each other.

Estimation of Nitrogen in Cell Suspensions

Nitrogen was estimated by the conventional micro-Kjeldahl technique.

Ultraviolet Absorption

This was used as a test for the presence of nucleic acid. The optical density of extracts was measured in 10-mm cells at 263 mμ by means of a Hilger 'Uvispek' spectrophotometer (Hilger & Watts Ltd., London, England). While this method of nucleic acid detection is not specific, it has the advantages that it is sensitive, simple, and does not involve loss of sample. In view of the very small amounts of material at our disposal it therefore seemed the best method to use.

Reagents

All reagents were AnalaR grade. The ^{32}P-labelled phosphate was obtained from the U.K.A.E.A. Laboratories, Amersham, England, as carrier-free orthophosphate in dilute HCl with a specific activity greater than 1000 c/g P. This solution was neutralized with 0.1 *N* NaOH and diluted to 10 μc/ml before use.

Results

Freshly harvested, well-washed, *Nitrobacter* cells were extracted as described and the phosphate contents of the extracts determined (Table I). Since the alkali-soluble fraction contained more phosphate than any other it was decided to concentrate on the analysis of this.

TABLE I

The distribution of phosphate in various fractions of *Nitrobacter* cells
(Phosphate compounds present in the cells were fractionated as described and the phosphate contents of the extracts determined. The final residue remaining after treatment with dilute alkali was further extracted with 5% T.C.A. for 15 minutes at 95°. All phosphate contents are expressed as μg of phosphate phosphorus/mg of nitrogen in the original cell suspension)

Fraction	Phosphate content (μg phosphate P/mg N)	
Original cell suspension	Total phosphate	167
Cold T.C.A.-soluble	Orthophosphate	6
	Acid-labile phosphate	3
	Total phosphate	11
Ethanol plus ethanol:ether-soluble	Total phosphate	52
Alkali-soluble	Orthophosphate	0
	Acid-labile phosphate	61
	Total phosphate	84
Final residue	Total phosphate	19
Final residue after hot T.C.A. extraction	Total phosphate	14

It apparently contained both acid-labile and acid-stable phosphate (Table I). In order to separate the acid-labile from the acid-stable compounds, 2 ml of the fraction was shaken with 0.1 g of Norit Ultra activated charcoal. This removed most of the acid-stable material and markedly reduced the optical density at 263 mμ (Table II); this table also shows the effect of T.C.A. in precipitating phosphate before and after treatment of the fraction with Norit. When a sample of the Norit-treated fraction was brought to pH 4.5 with 0.1 *M* acetate buffer a purple color developed on the addition of a few drops of

TABLE II

Effects of Norit and trichloracetic acid on the phosphate content and ultraviolet absorption of the alkali-soluble fraction of *Nitrobacter* cells

(Cells were extracted as described and the phosphate content and absorption at 263 mμ of the alkali-soluble fraction were determined. Part of the fraction was shaken with Norit Ultra charcoal (0.05 g/ml of extract), the charcoal centrifuged, and the phosphate content and the absorption at 263 mμ again determined. Part of the untreated fraction and part of the Norit-treated fraction were each added to an equal volume of 10% T.C.A., the precipitate centrifuged and the phosphate content and absorption at 263 mμ of each supernatant determined. All samples were diluted 15-fold for the measurement of optical density at 263 mμ in a 10-mm cell. Phosphate contents are expressed as in Table I. Orthophosphate was absent from all the samples)

Fraction	Phosphate content (μg phosphate P/mg N)		Optical density at 263 mμ
Untreated alkali-soluble	Acid-labile phosphate	64	1.052
	Total phosphate	84	
Norit-treated	Acid-labile phosphate	58	0.395
	Total phosphate	65	
Treated with T.C.A.	Acid-labile phosphate	19	0.340
Treated with Norit and then with T.C.A.	Acid-labile phosphate	47	0.038
	Total phosphate	52	

toluidine blue (30 mg/liter); this positive "metachromic reaction" indicated the presence of polyphosphate. Chromatography of the Norit-treated fraction by the method of Crowther (6) also revealed a spot that travelled at the same rate as the sodium hexametaphosphate used as a marker thus further indicating the presence of polyphosphate. Neither orthophosphate nor pyrophosphate was found in the Norit-treated fraction.

Table III shows the uptake of orthophosphate from the medium into the various phosphate fractions when *Nitrobacter* was incubated aerobically with

TABLE III

Uptake of orthophosphate by *Nitrobacter* during incubation with nitrite

(Cells were incubated with aeration at 30° for 45 minutes with 25 m*M* nitrite in 0.03 *M* phosphate buffer, pH 7.5, in order to stabilize the rate of phosphate exchange between cells and suspension medium. ^{32}P-labelled orthophosphate was then added to give a specific activity of 447 counts/minute/μg phosphate P (determined as described) and incubation continued for a further 30 minutes. The cells were then washed with ice-cold water and suspended in 5% T.C.A. at 0°. Phosphate compounds were extracted as before. The phosphate content and activity of each extract was determined as described; phosphate contents and phosphate uptakes are both expressed as μg phosphate P/mg of nitrogen in the original cell suspension. The phosphate uptakes were calculated on the assumption that the uptake of 1 μg P by any fraction would increase the radioactivity of that fraction by 447 counts/minute)

Fraction	Phosphate content (μg phosphate P/mg N)		Phosphate uptake in 30 min (μg phosphate P/mg N)
Original cell suspension	Total phosphate	200	23
Cold T.C.A.-soluble	Orthophosphate	17	
	Acid-labile phosphate	2	
	Total phosphate	23	11
Ethanol, ethanol:ether-soluble	Total phosphate	35	1
Alkali-soluble	Orthophosphate	0	
	Acid-labile phosphate	66	
	Total phosphate	80	9
Final residue	Total phosphate	50	2

25 m*M* sodium nitrite in 0.03 *M* phosphate buffer, pH 7.5, containing ^{32}P-labelled orthophosphate. When this experiment was repeated using cells that had been previously incubated for 3 hours at 30° with 25 m*M* nitrite in 0.03 *M* borate buffer, pH 7.9, the rate and pattern of uptake of orthophosphate was not significantly different from that shown in Table III.

Discussion

This preliminary investigation has revealed the general pattern of phosphate compounds occurring in *Nitrobacter* cells.

The organism takes up orthophosphate at a very low rate during oxidation (Table III), which supports the findings of Simpson (1). Most of the phosphate was taken into the cold T.C.A.- and alkali-soluble fractions, but the uptake into the T.C.A.-soluble fraction was probably largely due to an exchange between orthophosphate in the cells and in the incubation medium.

The fraction with the largest phosphate content was the alkali-soluble fraction which was also notable for its high proportion of acid-labile phosphate (Table I). The acid-stable phosphate in this fraction was probably nucleic acid. Although some nucleic acid may have been hydrolyzed by treatment with 1 *N* HCl for 7 minutes at 100°, it seems unlikely that hydrolysis of nucleic acid could account for all the acid-labile phosphate found in the alkali-soluble fraction since Winder and Denneny (7) found that only 23% of ribonucleic acid phosphate and 14% of deoxyribonucleic acid were liberated under such conditions of hydrolysis. Moreover, when the alkali-soluble fraction was treated with Norit it was noted (Table II) that while the total phosphate content, and the optical density at 263 mμ, were reduced (indicating that nucleic acid had been removed) only a little of the acid-labile phosphate was lost. The U.V. absorption at 263 mμ shown by the Norit-treated fraction may well have been due to protein since subsequent treatment with T.C.A. further reduced the U.V. absorption without greatly affecting the phosphate content (Table II). Treatment of the alkali-soluble fraction with 5% T.C.A. without prior Norit treatment markedly reduced the acid-labile phosphate; this suggests that the acid-labile phosphate was coprecipitated by the T.C.A. with the material removed by the Norit.

These results indicate that the alkali-soluble fraction contains some totally acid-labile compound and, since this compound behaved during extraction like the polyphosphate (metaphosphate) of yeast (8) it seems likely that it is also a polyphosphate. This conclusion is supported by the metachromatic reaction of the compound with toluidine blue (8) and by its chromatographic behavior. It is of interest that Knaysi (9) found that the chemosynthetic autotroph *Thiobacillus thio-oxidans* also contains a polyphosphate.

Acknowledgments

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THE PULSE RATE AS A MEANS OF MEASURING METABOLIC RATE IN MAN[1]

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Abstract

The possibility of using the pulse rate as a means of measuring metabolic rate in man has been investigated. The relation between pulse rate and metabolic rate was initially established for six subjects by a standard technique over the range of activity from lying still to moderate exertion. The reliability of the relation was tested by repetition of the measurements, and, in the range of muscular exercise, by comparing values for metabolic rate computed from the pulse rate with measured values. While a subject is lying, sitting, and standing still the pulse rate is too variable to be useful for measuring metabolic rate. During muscular work, however, there is a consistent relation and, with certain qualifications, this can provide a practical method of measuring metabolic rate.

Introduction

A previous investigation (1) called for repeated measurements of the metabolic rates of a number of individuals while they were lying, sitting, and standing, and while they were engaged in their everyday occupations. The methods of measuring metabolic rate in common use impose some constraint on the subject, and involve gas analyses which are laborious to carry out. It is particularly difficult to measure the energy spent in ordinary daily activities, which account for a considerable part of the total energy metabolism and are too variable for "time-and-activity" records and sample measurements of oxygen consumption to be entirely satisfactory. There is a need for a method of measuring metabolic rate continuously over long periods, with minimal disturbance to the subject, and without excessive labor on the part of the investigator. Existing knowledge suggested that measurement of the pulse rate might provide a possible method. The relation between pulse rate and metabolic rate has accordingly been reinvestigated from the point of view of its practical usefulness. This paper briefly reviews the existing information and presents the results of some experiments carried out to test the usefulness of the relationship.

As early as 1907 Benedict (2) reported that changes in the pulse rate could be correlated with changes in heat production in any one individual. Benedict and his associates confirmed and extended this observation in later work (3–6), and suggested that the pulse rate might provide a practical and satisfactory way of measuring total metabolism. Lindhard (7) observed concomitant changes in pulse rate and oxygen consumption when he compared the physiological effects of lying, sitting, and standing. Murlin and Greer (8) confirmed that pulse rate and oxygen consumption were related. Henderson

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and Prince (9), in a more detailed study, showed that, in any one individual, one relation between pulse rate and oxygen consumption held good during rest and very light activity, and there was an abrupt transition to a second relation when activity was increased to exercise. The relation during exercise was found to be the more constant and linear, though it in turn broke down at high levels of exertion.

Later studies have been mainly concerned with the relationship during exercise. It has been repeatedly confirmed that there is a linear relation between an individual's pulse rate and oxygen consumption during submaximal muscular work (10–20). It has, however, been found that the relationship may be different for activities employing different groups of muscles, and according to whether the muscular effort is "dynamic" or "static" (12, 17, 18, 21). Training lowers the pulse rate at any level of metabolism, for the type of work practised and for as long as training continues (11, 14, 17). It has been suggested that the steepness of slope of the graph is an inverse measure of capacity for work (22). Fatigue, or failure to maintain an adequate circulation to working muscles, causes the pulse rate to rise as work continues at a constant level (18, 23). After strenuous exercise the pulse rate remains high after oxygen consumption has returned to normal (24). Posture affects both resting and working pulse rates (15, 25–27). Changes in environmental temperature cause changes in pulse rate which may or may not be accompanied by changes in metabolic rate, and dehydration raises the pulse rate (19, 28–32). Food, drink, and smoking may affect the relationship in varying degrees (33), and after meals pulse rate rises more than oxygen consumption (18).

Despite the difficulties these observations raise, some previous workers have considered that the pulse rate might be a useful means of measuring metabolic rate, under controlled experimental conditions, and provided that the relation has been established for the individual subjects and for the type of work and circumstances of the experiment (3, 18, 19, 32).

Methods

Two women and four men were employed as subjects. Their measurements are given in Table I. All were members of the laboratory staff and were accustomed to this kind of work. The experiments were carried out with the subjects in the postabsorptive state, and in the laboratory experiments the temperature was 20° C. Only the subject and observer were present during experiments.

For each subject the relation between pulse rate and metabolic rate was first established by a standard technique for lying, sitting, standing, and three rates of work on a bicycle ergometer. The set of measurements for each individual was made during one morning, in ascending order of rates of work. When the subjects were lying, sitting, or standing, the pulse rate was measured at 5-minute intervals, and after a steady pulse rate had been found in two successive counts, expired air was collected for 10 minutes. During the collection, the pulse rate was counted three times, at 3-minute intervals. When the

TABLE I

Particulars of the experimental subjects

Subject	Sex	Age	Height (cm)	Weight (kg)	Surface area (m^2)*
1	F	37	160	54.3	1.56
2	F	38	164	61.8	1.68
3	M	57	174	52.3	1.66
4	M	24	185	86.8	2.11
5	M	23	183	78.0	2.00
6	M	31	172	52.7	1.62

*By Du Bois' formula.

subjects were standing they were made to move their legs from time to time in order to prevent pooling of blood in their legs (26). On the bicycle ergometer the rates of working were chosen, for each subject, to give a difference in pulse rate of about 10 beats per minute between successive levels of work. The subjects pedalled at a steady pace with the help of a metronome. After the subject had been pedalling steadily for at least 5 minutes, expired air was collected for 10 minutes. The pulse rate was again counted three times, at 3-minute intervals, during the collection. This initial set of measurements was plotted as a "calibration" graph for each individual.

To test the constancy of the results, the measurements for lying, sitting, and standing for four subjects were repeated up to 10 times. Further measurements were also made during work on the bicycle ergometer and during other forms of muscular activity in two subjects (Nos. 4 and 5). The activities included stepping on and off a stool 12 in. high at fixed rates, walking outdoors on a level track, and cycling outdoors on a level track. Subject 4 was made to step on and off the stool at rates of 12 and 24 steps per minute, and subject 5 at rates of 6, 9, 12, and 24 steps per minute. Walking and cycling were at the subject's natural comfortable pace.

Expired air was collected in Douglas bags (34, 35) when the subjects were lying, sitting, or standing. The volumes of expired air were measured with a calibrated dry gas-meter, and samples for analysis were collected in rubber containers (36). When the subjects were exercising, a Kofranyi-Michaelis respirometer, fitted with a standard Siebe-Gorman mask, was used to measure the volumes of expired air and collect the samples (36–38). The samples were analyzed with a Hartmann and Braun analyzer, which had been calibrated against Haldane's apparatus (39). Metabolic rates were calculated from Cathcart and Cuthbertson's tables (40), and expressed in kcal/m^2/min.

Pulse rates were usually counted by palpation of the radial pulse for 1-minute periods, timed with a stop watch. The three counts taken during each collection of expired air were averaged. When such counting was impossible, during walking and cycling outdoors, the first 10 beats immediately after cessation of the activity were timed (18, 19, 41). An electronic pulse-rate meter was also used for some of the measurements. This used electrocardiographic potentials, and was made to the design of Boyd and Eadie (42) with certain modifications. It was found necessary to redesign the multivibrator stage of the circuit to make the calibration independent of the shape and size of the *R* waves of the

electrocardiogram; an electromagnetic counter was provided as well as a visual rate-meter; and several monitoring points were provided in the circuit and used in conjunction with an oscilloscope to set the controls to the optimum for each subject.

Results

Figure 1 shows the relation between pulse rate and metabolic rate found for subject 5 in the initial measurements. The other subjects produced similar graphs. As has been previously described, the relation between pulse rate and

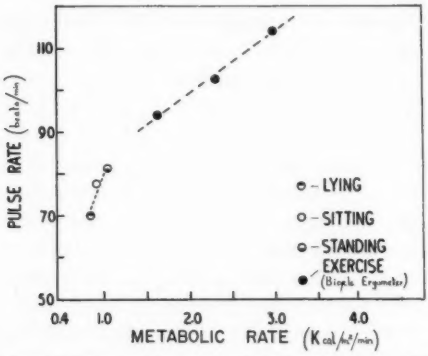

FIG. 1. The relation between pulse rate and metabolic rate while a subject is lying, sitting, standing, and performing three levels of work on a bicycle ergometer: subject 5.

metabolic rate appears to consist of two parts, both approximately linear; one applies over the range of activity covered by lying, sitting, and standing, and the other from light to submaximal muscular work. The change in slope occurred at pulse rates which varied in different individuals from 60 beats per minute for subject 3 to 100 for subject 1. The calibration points obtained during exercise lay almost on a straight line in all subjects. In the lying, sitting, and standing range, however, the three calibration points did not allow a straight line to be drawn with the same confidence.

Previous workers have stressed the differences between individuals in the relationship, and this has been confirmed. At a metabolic rate of 2 kcal/m²/min, for instance, subject 3 had a pulse rate of 80, subject 6 of 90, subject 5 of 100, and subject 1 of 115 beats per minute.

In the lower range of activities the results from the same subject varied on different occasions. Table II lists the average values for pulse rate and metabolic rate found in repeated measurements on four of the subjects while they were lying, sitting and standing, together with the ranges of values found. Although each set of mean values shows a fairly linear relationship and a significant correlation between pulse rate and metabolic rate, considerable ranges of readings for both quantities were often obtained for the same activity, even though the experimental technique had been carefully standardized. The variations in pulse rate and metabolic rate at any one level of activity did not

TABLE II

Average values and ranges of pulse rate and metabolic rate during lying, sitting, and standing

Subject	Lying			Sitting			Standing		
	Pulse rate (beats/min)	Metab. rate (kcal/m²/min)	No. observ-ations	Pulse rate (beats/min)	Metab. rate (kcal/m²/min)	No. observ-ations	Pulse rate (beats/min)	Metab. rate (kcal/m²/min)	No. observ-ations
1	68 (67–70)	0.69 (0.65–0.73)	4	69 (67–70)	0.79 (0.70–0.84)	4	81 (80–82)	0.86 (0.82–0.90)	4
3	48 (44–59)	0.43 (0.39–0.46)	9	52 (52–52)	0.46 (0.45–0.46)	2	78 (76–80)	0.53 (0.48–0.58)	4
4	63 (59–66)	0.58 (0.54–0.65)	10	73 (71–76)	0.78 (0.74–0.83)	3	86 (85–87)	1.03 (0.91–1.16)	5
5	71 (67–77)	0.82 (0.76–0.89)	8	81 (77–84)	0.93 (0.90–0.96)	3	83 (82–85)	1.02 (0.98–1.10)	3

appear to be closely correlated, and no subject gave consistent readings at all three levels of activity. The limits of confidence of prediction of metabolic rate from pulse rate exceeded $\pm 10\%$ of the sitting metabolic rate in all four subjects.

During exercise the relationship was more consistent. Figures 2 and 3 show pulse rates plotted against metabolic rates for two subjects performing different kinds of work at various rates. The lines in the figures were drawn from the

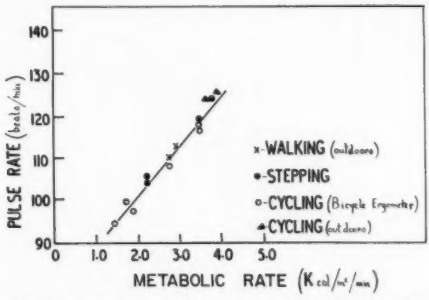

FIG. 2. The relation between pulse rate and metabolic rate during various types of muscular work: subject 4.

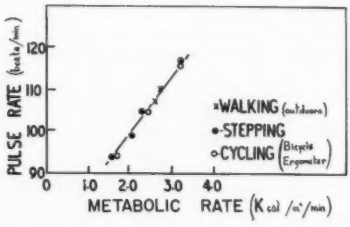

FIG. 3. The relation between pulse rate and metabolic rate during various types of muscular work: subject 5.

initial calibration measurements obtained for the individual on the bicycle ergometer; the points represent subsequent measurements which included this and other kinds of activity. Both subjects show a consistent relation between pulse rate and metabolic rate, despite repetition of measurements and variation in the type of work. The constancy of the relation is of the same order as was found by Berggren and Christensen (19).

Figures for metabolic rate computed from the pulse rates, using the line drawn from the initial set of measurements as a calibration, are compared with simultaneously measured values in Table III. The table gives the discrepancies between the predicted and measured values as percentages of the measured values. In 14 comparisons made on one subject, the discrepancy reached 12%; in 9 comparisons on the other subject it did not exceed 5%. Statistical calculation shows the highly significant correlation coefficients between pulse rate and measured metabolic rate of 0.98 for subject 4, and 0.99

TABLE III

Comparison between values of metabolic rate found (*a*) by measurement and (*b*) by computation from pulse rate, in two subjects doing muscular work

	Subject 4				Subject 5			
		Metabolic rate (kcal/m²/min)				Metabolic rate (kcal/m²/min)		
Activity	Pulse rate	(*a*) Measured	(*b*) Computed	Discrepancy	Pulse rate	(*a*) Measured	(*b*) Computed	Discrepancy
Cycling, bicycle ergometer	95	1.43	1.46	+ 2%	94	1.67	1.60	−4%
	97	1.88	1.72	− 9%	105	2.42	2.40	−1%
	99	1.72	1.92	+12%	116	3.21	3.19	−1%
	108	2.74	2.61	− 5%				
	116	3.48	3.32	− 5%				
	118	3.45	3.44	0%				
Stepping	104	2.23	2.28	+ 2%	94	1.60	1.60	0%
	106	2.24	2.40	+ 7%	99	2.00	1.96	−2%
	119	3.48	3.60	+ 3%	105	2.28	2.40	+5%
	125	3.74	3.95	+ 6%	117	3.29	3.26	−1%
Walking	110	2.78	2.80	+ 1%	107	2.59	2.50	−3%
	113	2.94	3.00	+ 2%	110	2.70	2.76	+2%
Cycling, outdoors	124	3.64	4.00	+10%				
	126	3.87	4.08	+ 5%				

for subject 5. The mean discrepancies between predicted and measured metabolic rates do not differ significantly from zero, i.e., the calibration did not change significantly between the sets of measurements. The 95% confidence limits for prediction of metabolic rate from pulse rate are ± 0.31 kcal/m²/min for subject 4, and ± 0.13 kcal/m²/min for subject 5.

Discussion

The variability of pulse rate found in the "quiet occupations" seems to preclude the use of pulse rate to measure metabolic rate in this range of activity. It is probable that the effect of metabolism on pulse rate is being confounded with the effects of other variables, such as the changes of posture (26, 27). Since many of a person's ordinary daily activities are in this range, the original hope that pulse rate would provide a useful means of measuring the energy metabolism of the "daily round" has had to be abandoned.

On the other hand, the consistent relation found during moderate exercise suggests that at this level of activity a pulse-rate method can be valid if proper precautions are taken. Its reliability would appear to depend largely on how far effects of other factors which influence pulse rate can be controlled. Among such factors are the degree of training for the activity in hand, the state of body temperature regulation, and the time since previous meals. The type of activity, and posture, are important. The results suggest that activities can be interchanged to a certain extent, but in all those studied the body was upright and work was performed predominantly with the legs. Emotional factors might also have to be considered. Where all these things can be satisfactorily controlled, the accuracy as judged by the agreement with gas analysis methods is quite good, and should be sufficient for many purposes. In view of the differences between individuals, which may reflect their differing capacities for work, it appears necessary to "calibrate" each individual separately, performing a suitably chosen activity in circumstances similar to those in which measurements are wanted. This can be done in a morning's work. A relation based on the average results from a group could not usefully be transferred to any one individual. The pulse rate method should be particularly useful when observations are wanted over an extended period of time; for example, for such an experiment as following the metabolism of a cyclist coping with a hilly course and varying winds. Furthermore, where the pulse rate is being partly determined by other factors than metabolism, for instance temperature, it is still a valuable measurement in its own right, and a better indication of the physiological "load" or "strain" the situation imposes than a measurement of metabolic rate alone.

The pulse rate can conveniently be recorded by an electrical counter using the electrocardiogram. The electrodes can be attached to the subject's chest, and cause less disturbance than the mask or mouthpiece required to collect expired air. The equipment used for this investigation was not portable. A number of designs of cardiotachometer are now available, however, and some of these are very compact and portable (43). In a portable equipment changing pulse rates can be recorded by a printing electromagnetic counter. Altern-

atively, preliminary work with the present equipment suggests that a fairly simple mechanical computer can be made to convert the pulse beats in each minute into the appropriate number of calories, according to the individual's calibration graph, and sum the calories. Remote recording via a radio link (44) is another alternative.

Acknowledgments

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AMINO ACID ACCUMULATION IN EHRLICH ASCITES CARCINOMA CELLS[1]

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Abstract

Measurements of the transport of amino acids into Ehrlich ascites carcinoma cells have shown that the following relationship exists between the intracellular steady-state concentration of the amino acid (C_z) and the extracellular concentration (C_0):

$$\frac{1}{C_z} = \frac{E_m}{C_m} \cdot \frac{1}{C_0} + \frac{1}{C_m}$$

where C_m is the maximum intracellular concentration (formed when C_0 is large) and E_m is a constant. It is shown that E_m is identical with K_m, the Michaelis constant, if a carrier enzyme is involved in the process of active transport and is, therefore, a measure of the affinity of the amino acid for the effective agent involved in the transport phenomenon.

The ratio of the steady-state intracellular and extracellular concentrations of amino acids exceeds unity with all amino acids examined. The responses of L-S-ethylcysteine transport to changes of potassium ion concentration and to changes of temperature differ from those of glycine transport and indicate that different carriers are involved in the active transport of these amino acids into Ehrlich ascites cells. This conclusion is supported by the fact that, whereas glycine and L-serine compete with each other for concentrative uptake, such mutual competition does not occur between S-ethylcysteine and glycine or L-serine or L-leucine.

Effects of the metabolic inhibitors, 2,4-dinitrophenol, iodoacetate, and stilbestrol show that these substances exercise inhibitory effects on active transport of amino acids by suppression of respiratory or glycolytic energy. Stilbestrol, which is a particularly potent inhibitor, is more effective under aerobic conditions (in the absence of glucose) than under anaerobic conditions (in the presence of glucose). It is reasonable to account for these results on the hypothesis that the carrier responsible for amino acid transport is ATP dependent and that the carrier breaks down, and is no longer available for amino acid transfer, if the ATP content of the cell is depleted.

It has been known since the early work of Van Slyke and Meyer (1) that amino acids may be held in animal tissues in much larger concentrations than in the plasma, and that isolated tissues may take up amino acids against a concentration gradient (2–8). Active transport, or transport against a concentration gradient, of both sugars and amino acids in the intestine has been well established (9, 10, 11). Isolated tumor cells show active transport of ions, sugars, and amino acids (12, 13, 14), the results of Christensen *et al.* (14) showing that glycine may be concentrated in vitro in Ehrlich ascites carcinoma cells more than 12 times the concentration in the medium. Moreover, amino acids not normally found in the animal body (15) may be concentrated in these cells. The active transport of glycine is suppressed by the presence of cyanide, sodium azide, or by anaerobiosis, indicating that the process is dependent on respiratory energy (16, 17, 18). Glycolytic energy, however, even under strictly anaerobic conditions, will enable Ehrlich ascites carcinoma cells to take up amino acids against a concentration gradient (19).

Inhibition of amino acid transport by other amino acids into Ehrlich ascites cells has been demonstrated. Thus DL-alanine (14) and sarcosine (20) inhibit

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concentrative uptake of glycine possibly because the two compounds compete with glycine for the same transport carrier. It has been suggested (21) that the exchange between various amino acids (sarcosine, N-acetylglycine, DL-diaminobutyrate, DL-alanine) and glycine in Ehrlich ascites carcinoma cells is due to a reaction between these amino acids and carrier-bound glycine. Active transport of an amino acid into a cell may thus consist of carrier-controlled reactions, consisting of either combination of an amino acid with a cell carrier or of an exchange between an amino acid and a carrier – amino acid complex.

It has been demonstrated (22) that the presence of L-S-ethylcysteine brings about a suppression of active transport of glycine into Ehrlich ascites carcinoma cells. It was also pointed out (22) that the presence of S-ethylcysteine inhibits the incorporation of glycine into the proteins of Ehrlich ascites carcinoma cells. L-S-ethylcysteine is, itself, actively transported into the ascites cell.

It is the purpose of this communication to describe some of the factors influencing amino acid transport and amino acid interrelations in Ehrlich ascites carcinoma cells.

Methods and Materials

The radioactive amino acids used in this study were obtained from Merck and Company, Limited, and from the Atomic Energy Commission of Canada. The radiopurities of glycine-1-C^{14}, leucine-1-C^{14}, and serine-3-C^{14} were established by chromatography.

The tissue used was an ascites tumor obtained from Dr. J. C. Hunter, of the National Institutes of Health, Bethesda, Maryland. The tumor was grown in male Swiss white mice and was of the Lettre strain of Ehrlich ascites tumor as developed by Dr. Hauschka at the Lankenau Institute of Philadelphia, Pa.

Preparation of Cells

The mice were used 6–10 days after injection of the tumor. The animals were sacrificed by cervical dislocation. An incision was made through the abdominal wall and the ascitic fluid was removed with a Pasteur pipette. Quite often the ascitic fluid contained a large number of blood cells. The contaminating blood elements were removed by spinning the ascitic fluid at 300 *G* for 30 seconds. This sedimented the tumor cells while leaving the blood cells in suspension. The tumor cells were resuspended in a Ringer medium of the following composition: NaCl = 128 m*M*, KCl = 5 m*M*, $CaCl_2$ = 3 m*M*, and $MgSO_4$ = 1.3 m*M* (with 10 m*M* sodium phosphate buffer, pH 7.4, for aerobic experiments and 25 m*M* sodium bicarbonate solution for anaerobic experiments), and this operation was repeated. By this procedure, relatively pure ascites cells could be obtained. They were then centrifuged at 300 *G* for 2½ minutes, and the "packed cell" volume measured. The cells were then resuspended in the Ringer medium, and 1 ml of suspension was added to each incubation vessel. The final cell suspension was made such that 1 ml of suspension contained 0.1 ml of packed cells.

Incubation of Tissue

Incubations were carried out in 25-ml Erlenmeyer flasks at 37° C with air as the gas phase, the cells being suspended in the Ringer medium with the addi-

tion of 10 m*M* phosphate buffer at pH 7.4. In experiments where respiration or glycolysis was measured the conventional Warburg apparatus was used. Sometimes the experiments were carried out under anaerobic conditions (the gas phase being purified N_2) without adding any substrate other than the test amino acid to the incubation medium. The test amino acid was added to the side arm of the Warburg vessel and tipped into the main compartment after 15 minutes preincubation at 37° C. Solutions of any other additions were tipped in from the side arm after the preincubation period. The final volume incubated was 3 ml.

Measurement of Amino Acid Uptake

After the incubation, the contents of the incubation vessels were poured into centrifuge tubes containing 5 ml of the ice-cold Ringer medium. The cells were centrifuged and the supernatant decanted. The cells were then suspended in 3 ml of 80% ethanol containing 1 ml of 1 *N* HCl per 100 ml ethanol. The suspension was centrifuged after 1 hour and 0.5 ml of the supernatant was placed on aluminum plates, dried, and the radioactivity measured. This technique involves washing the cells once in ice-cold Ringer medium. This washing is intended to remove any amino acids adsorbed onto the cell surface and to minimize the error due to the presence of radioactive amino acids trapped in the packed cell mass. Since most amino acids are known to diffuse quite rapidly through the cell membrane, this washing must be done very rapidly (at 0° C) to reduce the possibility of removing any intracellular amino acids. One washing reduces the final estimation by 5–10%; two washings may cause a decrease of 15–20%. The reduction after the first washing is probably due mostly to removal of contaminating amino acids, but, because of the time required, the loss after two washings probably includes some amino acids which diffused out of the cell. The technique adopted, therefore, included only one washing.

It has been shown (17, 26) that when Ehrlich ascites carcinoma cells are incubated with glycine-1-C^{14} the steady-state level is reached within 30 minutes under the given experimental conditions. This also occurs with the other radioactive amino acids used in this work.

Measurement of Radioactivity

The samples to be assayed were placed on aluminum plates with an area of approximately 5 cm^2. The radioactivity was measured with a thin mica window Geiger–Müeller tube attached to a Tracerlab shielded sample changer and automatic scaler.

Results

The ability of Ehrlich ascites cells to transport glycine, serine, and S-ethylcysteine was measured. The results obtained are recorded in Table I. It can be seen from this table that L-serine is the most actively transported of the amino acids used. At equilibrium, under the given experimental conditions the accumulation of serine is 2.08 μmoles/0.1 ml cells, and the concentration distribution ratio (intracellular/extracellular) is 10.4/1. The accumulation of

TABLE I

Amino acid transport into Ehrlich ascites cells

Amino acid (0.002 *M*)	Amino acid transport*	Distribution† ratio
Glycine-1-C^{14}	1.55	7.75/1
L-Serine-3-C^{14}	2.08	10.4/1
L-S^{35}-ethylcysteine	0.74	3.2/1

NOTE: The incubation was allowed to take place for 1 hour at 37° C in Ringer phosphate solution with air as the gas phase. The radioactivity of the amino acids per 3 ml was: glycine-1-C^{14}, 1.09×10^5 c.p.m.; L-serine-3-C^{14}, 0.85×10^5 c.p.m.; L-S^{35}-ethylcysteine, 1.14×10^5 c.p.m.

*Amino acid transport is expressed as μmoles/0.1 ml packed cells.

†Distribution ratio is defined as: $\frac{\text{concn. of intracellular amino acid}}{\text{concn. of extracellular amino acid}}$.

L-S-ethylcysteine is, at equilibrium, 0.64 μmole/0.1 ml cells, and the distribution ratio is 3.2/1. Though this accumulation is quite small when compared with that of serine and glycine, it has been found to be at least equal to that of L-valine or L-leucine. The distribution ratio of all the amino acids tested, including L-S-ethylcysteine, was greater than 1. This means that some factor other than passive diffusion is contributing to the concentrative uptake of each of these amino acids.

The Effects of 2,4-Dinitrophenol, Iodoacetate, and Stilbestrol on Amino Acid Transport

The results of the addition of 2,4-dinitrophenol and of iodoacetate on the amino acid transport are recorded in Table II. It will be seen that 2,4-dinitrophenol (0.1 m*M*) inhibits the concentrative uptake of glycine by approximately

TABLE II

Effects of 2,4-dinitrophenol and iodoacetate on amino acid transport

Amino acid (0.002 *M*)	Inhibitor* added	Amino acid transport†	
		Aerobic	Anaerobic
Glycine-1-C^{14}	Nil	1.37	1.73
	2,4-Dinitrophenol	0.715	1.68
	Iodoacetate	1.68	0.60
L-S^{35}-ethylcysteine	Nil	0.56	0.67
	2,4-Dinitrophenol	0.32	0.66
	Iodoacetate	0.44	0.28

NOTE: One-hour incubation at 37° C in Krebs–Ringer medium. In aerobic experiments 0.01 *M* sodium phosphate buffer was used. In anaerobic experiments 0.025 *M* sodium bicarbonate and 0.01 *M* glucose were used. The radioactivities of the amino acids in 3 ml were: L-S^{35}-ethylcysteine, 1.30×10^5 c.p.m.; glycine-1-C^{14}, 1.09×10^5 c.p.m.

*2,4-Dinitrophenol concentration was 10^{-4} *M*; iodoacetate concentration was 6.7×10^{-4} *M*.

†Amino acid transport is expressed as μmoles/0.1 ml packed cells.

50% (in confirmation of earlier observations (18, 24)) and that of S-ethylcysteine by 40%. It has no significant effect on the transport of these amino acids under anaerobic conditions. Iodoacetate (0.67 m*M*) inhibits the anaerobic transport of glycine by 65% and that of S-ethylcysteine by 55%, and has no significant effect on the aerobic transport of these amino acids. Stilbestrol inhibits active transport of glycine to a greater extent under aerobic conditions than under anaerobic (Tables III and IV). The inhibition of transport seems

TABLE III

Effects of stilbestrol on aerobic glycine transport in absence of glucose with Ehrlich ascites carcinoma cells

Concentration of stilbestrol	μl oxygen consumed per 0.1 ml packed cells	Inhibition (%)	Steady-state concentration of glycine, μmoles per 0.1 ml packed cells	Inhibition (%)
Nil	90	—	1.46	—
0.002 m*M*	66	27	1.35	8
0.03 m*M*	48	47	0.87	40
0.06 m*M*	21.6	76	0.24	83
0.2 m*M*	4.75	95	0.18	88

NOTE: The stilbestrol was dissolved in methanol, and 0.5 ml of the methanol solution was diluted to 10 ml with water. The final methanol concentration in the incubating medium was 0.2 *M*, a concentration which is without effect on respiration, glycolysis, or glycine transport in Ehrlich ascites carcinoma cells. Incubation for 1 hour at 37° in Krebs–Ringer–phosphate medium. Radioactivity of glycine was 1.2×10^5 c.p.m. per 3 ml. Concentration of glycine = 2 m*M*.

TABLE IV

Effects of stilbestrol on anaerobic glycine transport in presence of glucose with Ehrlich ascites carcinoma cells

Concentration of stilbestrol	μl CO_2 evolved per 0.1 ml packed cells	Inhibition (%)	Steady-state concentration of glycine, μmoles per 0.1 ml packed cells	Inhibition (%)
Nil	145	—	0.95	—
0.03 m*M*	157	—	0.87	8
0.2 m*M*	114	21	0.52	44
0.3 m*M*	112	23	0.37	60

NOTE: Conditions as in Table III, except that incubation was for 1 hour anaerobically at 37° in Krebs–Ringer–bicarbonate medium, in the presence of glucose (0.01 *M*).

always under these circumstances to be accompanied by either inhibition of respiration or of glycolysis. These results are consistent with the conclusion that the concentrative uptake of both glycine and S-ethylcysteine requires energy which can be supplied by respiration or, under anaerobic conditions, by glycolysis.

Effects of Potassium Ions on Amino Acid Transport

Christensen and Riggs (16) have shown that a high potassium ion concentration leads to an inhibition of amino acid transport by Ehrlich ascites cells. They have also shown that the absence of potassium ions from the incubation medium results in a marked decrease in the rate of amino acid transport. It appears, therefore, that potassium ions are essential for active amino acid transport. Similar results were obtained by Riklis and Quastel (23) in their studies of the effect of potassium ions on active transport of glucose in guinea-pig intestine, sodium ions, however, being essential for this transport.

The dependence of glycine and S-ethylcysteine transport on potassium ion concentration was studied. The results obtained from these studies are recorded in Fig. 1. It will be seen from the results given in this figure that when

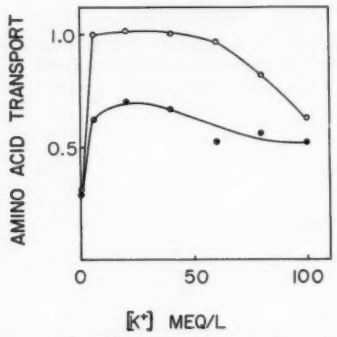

FIG. 1. The effect of potassium ions on the concentrative uptake of glycine and L-S-ethylcysteine.
Glycine ○——○, L-S-ethylcysteine ●——●. The transport is expressed as μmoles/0.1 ml packed cells/hour. The concentration of test amino acid was 2 m*M* and the activity of amino acid per incubation vessel was glycine-1-C^{14}, 0.95×10^5 c.p.m.; L-S^{35}-ethylcysteine, 0.5×10^5 c.p.m.

potassium ions are excluded from the incubation medium, the accumulation of both S-ethylcysteine and glycine is very low. Under these conditions, the accumulation of both glycine and S-ethylcysteine by ascites cells is 0.3 μmole/0.1 ml cells. At a potassium ion concentration of 5.6 meq/liter (the normal concentration in the incubation medium), the accumulation of glycine is increased to 1.0 μmole/0.1 ml cells. Further increase in potassium ion concentration to 50 meq/liter has very little effect on glycine transport, but when the potassium ion concentration is increased still further there is a progressive decrease in glycine uptake. At a potassium ion concentration of 100 meq/liter, the glycine accumulated after 1 hour of incubation is 0.85 μmole/0.1 ml cells.

At a potassium ion concentration of 5.6 meq/liter the L-S-ethylcysteine accumulated by the ascites cells in 1 hour is 0.62 μmole/0.1 ml cells. This stimulation of transport is maximal at a potassium ion concentration of 20 meq/liter when the S-ethylcysteine accumulation is 0.7 μmole/0.1 ml cells. Higher concentrations of potassium ion cause a decrease in S-ethylcysteine transport. The inhibition of accumulation is maximal at 60 meq/liter potassium ion. Further increase in potassium ion does not result in any further decrease in L-S-ethylcysteine transport. In this respect the effects of potassium ions on S-ethylcysteine and glycine transport differ. While the inhibition of S-ethylcysteine uptake is maximal at 60 meq/liter potassium ion, the glycine uptake (steady-state concentration) decreases at a fairly rapid rate even at a potassium ion concentration of 100 meq/liter. In general, however, the effects of potassium ion on L-S-ethylcysteine and glycine transport are similar, i.e., both systems require potassium ions for maximum activity and both systems are inhibited by high potassium ion concentrations.

The Effects of Temperature on Amino Acid Transport

The effects of change of temperature of incubation on glycine and S-ethylcysteine transport were compared. The results are recorded in Fig. 2. It will be seen that the transport of both glycine and S-ethylcysteine is quite small at

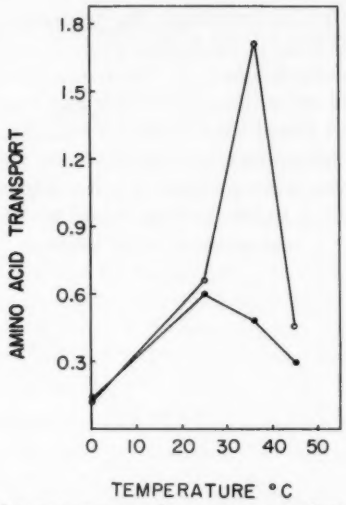

FIG. 2. The effects of temperature on the transport of glycine and L-S-ethylcysteine. Glycine ○——○, L-S-ethylcysteine ●——●. The transport is expressed as μmoles/0.1 ml packed cells/hour. The concentration of the test amino acid was 2 m*M* and the activity of amino acid per incubation vessel was glycine-1-C^{14}, 10^5 c.p.m.; L-S^{35}-ethylcysteine, 0.95×10^5 c.p.m.

0° C (0.14 μmole/0.1 ml cells). The Q_{10} between 10°–20° C is approximately 1.5. Increasing the temperature to 37° C causes a marked increase in the steady-state concentration of glycine (1.72 μmoles/0.1 ml cells). The Q_{10} for glycine transport between 25° C–37° C is 2.41. Further increase in temperature causes a decrease in the steady-state concentration and at 45° C the glycine accumulated in 1 hour is only 0.46 μmoles/0.1 ml cells.

The effects of increasing the temperature of incubation above 25° C, on the steady-state concentration of L-S-ethylcysteine transport, differ from those found with glycine. Above 25° C, increasing the temperature causes a decrease in the steady-state concentration of L-S-ethylcysteine transport. At 37° C the S-ethylcysteine accumulated in 1 hour is 0.48 μmole/0.1 ml cells and at 45° C it is only 0.3 μmole/0.1 ml cells. The optimum temperature for S-ethylcysteine transport seems to be 25° C.

The transport of both these amino acids shows a high degree of temperature dependence confirming the conclusion that the transport is a carrier-catalyzed process.

Considerations of Amino Acid Transport into Ehrlich Ascites Carcinoma Cells

Heinz (25) has measured rates of entry of glycine into Ehrlich ascites cells at varying external glycine concentrations. The uptake is very rapid, the theoretical half saturation value being reached in 3 minutes. By plotting the apparent influx against corresponding extracellular glycine concentration, a curve was obtained which fitted a Michaelis–Menten curve. The value of K_m (Michaelis constant) for glycine at 37°, i.e., the molar concentration of glycine required to give half the maximum rate of entry, was 3.7×10^{-3}.

In the experiments to be described, the transport process was allowed to proceed to equilibrium (1-hour incubation period) at 37° and the final concentration within the cell was determined. Such equilibrium intracellular concentrations of amino acid were compared with the corresponding extracellular concentrations. It was found that linear relationships are obtained between the reciprocals of the intracellular and extracellular amino acid concentrations when steady states have been reached. Typical results for glycine, L-proline, L-leucine, and L-serine are shown in Figs. 3 and 4. These relationships would

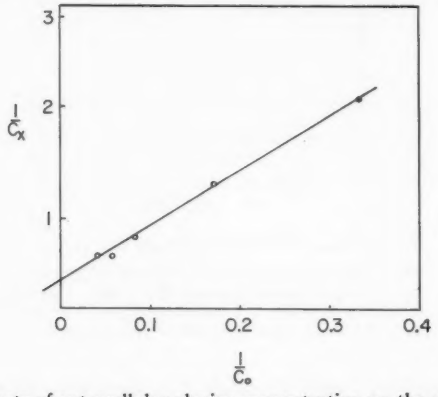

FIG. 3. The effects of extracellular glycine concentration on the steady-state concentration of glycine.

C_0 is the extracellular glycine concentration expressed as μmoles/incubation vessel and C_x is the intracellular glycine concentration after 1-hour incubation expressed as μmoles/0.1 ml packed cells. The specific activity of the glycine-1-C^{14} was 0.16×10^6 c.p.m./μmole.

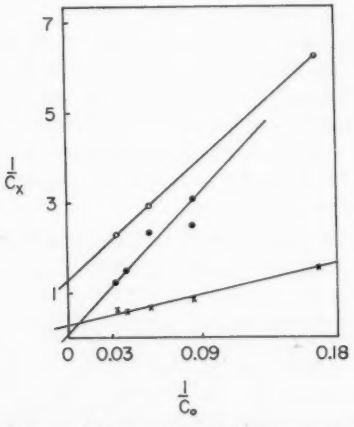

FIG. 4. The effects of extracellular amino acid concentration on amino acid steady-state concentration; ○——○, L-proline; ●——●, L-leucine; ×, L-serine. C_0 is the extracellular amino acid concentration expressed as μmoles/incubation vessel and C_x is the intracellular amino acid concentration after 1-hour incubation expressed as μmoles/0.1 ml packed cells. The specific activities of the amino acids were DL-leucine-1-C^{14}, 0.28×10^6 c.p.m./μmole; L-serine-3-C^{14}, 0.14×10^5 c.p.m./μmole and DL-proline-1-C^{14}, 0.38×10^6 c.p.m./μmole.

be expected if the amino acid transport results conform to the adsorption isotherm:

[1] $$C_x = \frac{C_m . C_0}{E_m + C_0} \text{ or } \frac{1}{C_x} = \frac{E_m}{C_m} . \frac{1}{C_0} + \frac{1}{C_m}$$

where C_x = intracellular, steady-state concentration of amino acid; C_0 = extracellular amino acid concentration; and C_m and E_m are constants. C_m = maximum intracellular concentration of amino acid (i.e. when C_0 is very large). E_m is the molar extracellular concentration of amino acid required to give half the maximum intracellular concentration of amino acid. The values of E_m, derived from the typical results given in Figs. 3 and 4, are:

for glycine $= 3.7 \times 10^{-3}$ M; for L-proline $= 7 \times 10^{-3}$ M;
for L-leucine $= 5.5 \times 10^{-2}$ M; for L-serine $= 6 \times 10^{-3}$ M.

Significance of E_m

It has been pointed out by Heinz (25) that, if the extracellular concentration of glycine is constant, the rate of transport of the amino acid follows the expression:

$$\frac{U_t}{U} = 1 - e^{-kt}$$

where U_t = uptake of amino acid in time t; U is the maximal uptake (i.e. equilibrium value); and t = time. Differentiation gives the expression:

$$\frac{dU_t}{dt} = U\,(ke^{-kt})$$

so that when $t = 0$,

[2] $$\frac{dU}{dt} = k . U$$

i.e. the initial rate of uptake of the amino acid is proportional to the equilibrium amount of glycine in the cell and thus to the equilibrium, or steady-state concentration.

Now (25) the apparent initial rate of uptake (V_c) of glycine is given by the expression:

[3] $$\frac{V}{V_c} = 1 + \frac{K_m}{C_0}$$

where V is the maximum initial velocity of uptake, C_0 = extracellular amino acid concentration, and K_m is a constant. K_m is formally identical with the Michaelis constant, if a carrier (enzyme) is involved in the phenomenon of active transport.

From equations [2] and [3] it follows that

$$\frac{V}{V_c} = \frac{U_{max}\ (\text{extracellular glycine concentration} = \infty)}{U_c\ \ (\text{extracellular glycine concentration} = C_0)} = 1 + \frac{K_m}{C_0}$$

where U_{max} and U_c are the corresponding steady-state levels.

But $$\frac{U_{max}}{U_c} = \frac{C_m}{C_x} = 1 + \frac{E_m}{C_0} \qquad \text{(from 1)}$$

Therefore $1+\frac{K_m}{C_0} = 1+\frac{E_m}{C_0}$.

Hence the value E_m is identical with K_m and is therefore a measure of the affinity of glycine for the effective agent involved in the transport phenomena.

It should be noted that, experimentally, E_m for glycine = 3.7×10^{-3} M and is identical with K_m for glycine transport found by Heinz (25). Moreover, further results have shown that E_m has the same value for glycine whether transport is measured under aerobic conditions or under anaerobic conditions in presence of glucose.

Results with L-S-Ethylcysteine

On plotting $1/C_x$ against $1/C_0$ for L-S-ethylcysteine the typical results shown in Fig. 5 are found. The relationship between $1/C_x$ and $1/C_0$ is only linear for concentrations below 8 mM. Above this concentration the plot curves toward

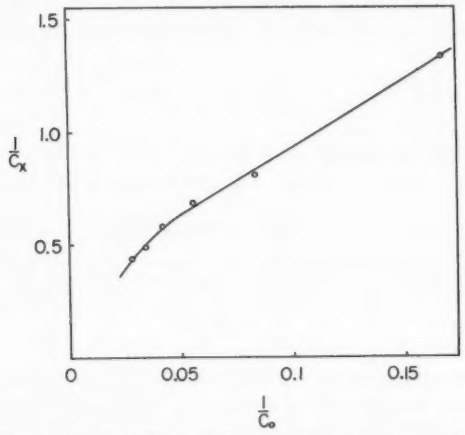

FIG. 5. The effects of extracellular L-S-ethylcysteine concentration on the steady-state concentration of L-S-ethylcysteine.

C_0 is the extracellular L-S-ethylcysteine concentration expressed as μmoles/incubation vessel and C_x is the intracellular L-S-ethylcysteine concentration after 1-hour incubation expressed as μmoles/0.1 ml packed cells. The specific activity of the L-S-ethylcysteine was 0.15×10^5 c.p.m./μmole.

the origin, indicating that at high concentrations the transport of L-S-ethylcysteine does not obey the adsorption isotherm. It would seem from these results that the rate of active transport of S-ethylcysteine greatly exceeds that of passive diffusion at concentrations below 8 mM but that above this concentration the rate of passive diffusion exceeds that of active transport.

Transport of Glycine and L-S-Ethylcysteine at 25°

Results recorded in Figs. 6 and 7 show the relationships between $1/C_x$ and $1/C_0$ at 25°. The E_m for glycine transport is calculated to be 6.9×10^{-3} M at 25°, a linear relationship holding between $1/C_x$ and $1/C_0$ at this temperature as at 37°. With L-S-ethylcysteine, the relationship at 25° is similar to that at 37°, the divergence from linearity occurring at 5 mM. Apparently, at concentrations

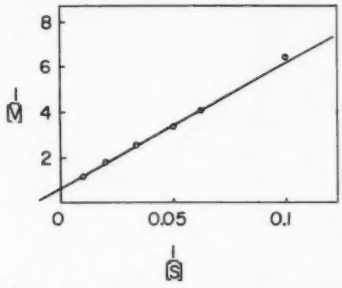

FIG. 6. The effects of extracellular glycine concentration on the transport of glycine at 25° C.

[S] represents the extracellular glycine concentration expressed as μmoles/incubation vessel. [V] represents the rate of glycine transport expressed as μmoles/0.1 ml packed cells/5 minutes. The specific activity of the glycine-1-C^{14} was 0.17×10^{5} c.p.m./μmoles. The incubation was carried out at 25° C for 5 minutes.

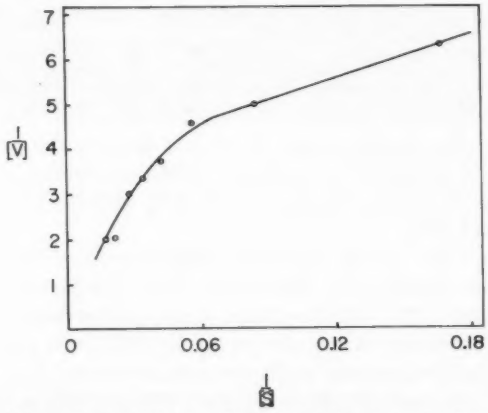

FIG. 7. The effects of extracellular L-S-ethylcysteine concentration on the transport of L-S-ethylcysteine at 25° C.

[S] represents the extracellular L-S-ethylcysteine concentration expressed as μmoles/incubation vessel. [V] represents the rate of L-S-ethylcysteine transport expressed as μmoles/0.1 ml packed cells/5 minutes. The specific activity of the L-S^{35}-ethylcysteine was 0.15×10^{5} c.p.m./μmole. The incubation was carried out at 25° C for 5 minutes.

above 5 m*M* at 25°, the process of passive diffusion exceeds in velocity that of active transport, so that the rate of accumulation of the amino acid approaches that of passive diffusion.

Competition between Amino Acids

The effects of L-serine, L-S-ethylcysteine, and glycine on each other's concentrative uptakes into Ehrlich ascites carcinoma cells were investigated. The results are recorded in Figs. 8, 9, 10.

Results given in Fig. 8 demonstrate the competition between glycine and serine for concentrative uptake. As will be seen, the maximum inhibition of uptake of 2 m*M* serine by glycine is about 40% at a glycine concentration of

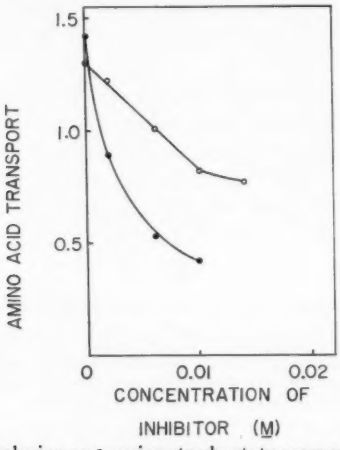

FIG. 8. The effects of glycine on L-serine steady-state concentration, and the effects of L-serine on glycine steady-state concentration.
Substrates: L-serine ○——○, glycine ●——●. The transport of the amino acids is expressed as μmoles/0.1 ml packed cells/hour. The activity of the glycine-1-C^{14} was 0.65×10^6 c.p.m./incubation vessel and that of the L-serine-3-C^{14} was 0.85×10^6 c.p.m./incubation vessel. The concentration of the test amino acids was 2 m*M*.

10 m*M*. L-Serine at a concentration of 10 m*M* inhibits the uptake of 2 m*M* glycine by 70%. At equimolar concentrations of L-serine and glycine (2 m*M*), serine inhibits glycine uptake approximately 40% while glycine inhibits serine uptake by less than 10%.

L-S-ethylcysteine has a very marked inhibitory effect on glycine uptake (Fig. 9). L-S-ethylcysteine, at a concentration of 10 m*M*, inhibits the uptake of 2 m*M* glycine by 77%. On the other hand, the maximum inhibition of 2 m*M* L-S-ethylcysteine uptake by glycine is only 20%, at a glycine concentration of 5 m*M*. Further increase in glycine concentration does not cause any further inhibition of S-ethylcysteine uptake. At equimolar concentrations, 2 m*M* L-S-ethylcysteine inhibits glycine uptake by 60% while glycine inhibits S-ethylcysteine transport only 10%.

The effects of S-ethylcysteine on L-serine uptake are recorded in Fig. 10. It will be seen that S-ethylcysteine at a concentration of 10 m*M* inhibits 2 m*M* L-serine uptake by 75%. L-Serine, on the other hand, at a concentration of 10 m*M*, inhibits the uptake of 2 m*M* L-S-ethylcysteine by only 30%. At equimolar concentrations (2 m*M*) the L-S-ethylcysteine inhibition of serine uptake is approximately 60%, while the L-serine inhibition of S-ethylcysteine uptake is only 15%.

The results obtained from a study of the mutual competition of L-leucine and L-S-ethylcysteine show that the latter amino acid (up to 10 m*M*) has no inhibitory effect on the uptake of the former amino acid (2 m*M*). L-Leucine, however, at 6 m*M* inhibits the uptake of L-S-ethylcysteine (2 m*M*) by 20%, no further inhibition occurring with increase of concentration of L-leucine. Two transport systems seem to be indicated, L-leucine having some affinity for that involving L-S-ethylcysteine.

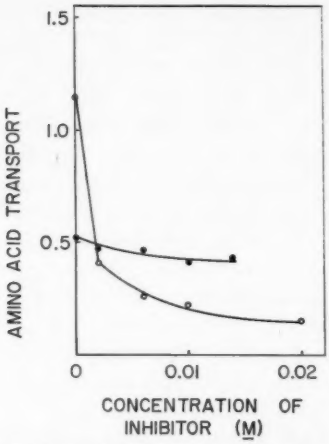

FIG. 9. The effect of L-S-ethylcysteine on glycine steady-state concentration, and the effect of glycine on L-S-ethylcysteine steady-state concentration.
Substrates: glycine ○——○; L-S-ethylcysteine ●——●. The amino acid transport is expressed as μmoles/0.1 ml packed cells/hour. The activity of the glycine-1-C^{14} was 0.65×10^5 c.p.m./incubation vessel and that of the L-S^{35}-ethylcysteine 0.26×10^5 c.p.m./incubation vessel. The concentration of the test amino acids was 2 m*M*.

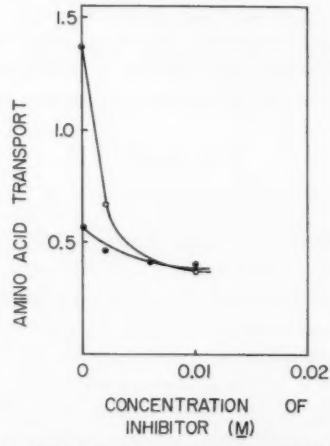

FIG. 10. The effect of L-S-ethylcysteine on L-serine steady-state concentration, and the effect of L-serine on L-S-ethylcysteine steady-state concentration.
Substrates: L-serine ○——○; L-S-ethylcysteine ●——●. The amino acid transport is expressed as μmoles/0.1 ml packed cells/hour. The activity of the L-serine-3-C^{14} was 0.19×10^5 c.p.m./incubation vessel and that of the L-S^{35}-ethylcysteine was 0.33×10^5 c.p.m./incubation vessel. The concentration of the test amino acids was 2 m*M*.

Discussion

Measurements of the transport into Ehrlich ascites carcinoma cells of glycine, L-serine, L-leucine, L-valine, and L-S-ethylcysteine have shown that serine is the most actively transported amino acid in this group. At equilibrium, under

the given experimental conditions, the ratio of intracellular to extracellular concentration of serine is 10.4/1. The ratio of intracellular and extracellular concentrations exceeds unity with all amino acids examined, a result that confirms the conclusion that some factor other than passive diffusion contributes to the concentrative uptake of the amino acids.

The optimal transport of L-S-ethylcysteine requires the presence of potassium ions, as is already known for glycine transport. Nevertheless, the response of the two amino acids to high concentrations of potassium ions is not identical. Inhibition of transport of L-S-ethylcysteine is maximal at 60 meq/liter potassium ions, but the steady-state concentration of glycine decreases at a fairly rapid rate even at potassium ion concentrations of about 100 meq/liter.

On comparing the effects of change of temperature on the transport of glycine and L-S-ethylcysteine, into Ehrlich ascites cells, notable differences are found. The temperature for the optimal steady-state concentration of glycine is 37° C whereas that for L-S-ethylcysteine is 25° C. The transport of both amino acids shows a high degree of temperature dependence as would be expected if transport is a carrier-catalyzed process. The results would indicate that glycine and L-S-ethylcysteine are transported by different carrier-controlled mechanisms, or if one carrier is involved, the carrier – amino acid complex shows different stabilities with change of temperature according to the nature of the amino acid.

A study has been made of the relationship between the intracellular steady-state concentration of amino acid (glycine, L-proline, L-leucine, and L-serine) in Ehrlich ascites cells and the extracellular concentration of the amino acid. This relationship conforms to an adsorption isotherm and may be expressed by the equation

$$\frac{1}{C_x} = \frac{E_m}{C_m} \cdot \frac{1}{C_0} + \frac{1}{C_m}$$

where C_x is the intracellular steady-state concentration, C_0 is the extracellular concentration, C_m is the maximum intracellular concentration (formed when C_0 is large), and E_m is a constant. It is shown that E_m is identical with K_m, the Michaelis constant, if a carrier enzyme is involved in the phenomenon of active transport, and is therefore a measure of affinity of the amino acid for the effective agent involved in the transport phenomenon.

The relationship between $1/C_x$ and $1/C_0$ in the case of L-S-ethylcysteine is linear only for concentrations below 8 mM. A departure from the adsorption isotherm occurs above this concentration, the results indicating that the rate of active transport of this amino acid exceeds that of passive diffusion only at concentrations below 8 mM. At 25° C this limiting concentration is 5 mM.

Studies of the effects of amino acids on each other's transport into Ehrlich ascites carcinoma cells show that, whereas apparently glycine and L-serine compete with each other for concentrative uptake, such mutual competition does not occur between glycine and L-S-ethylcysteine, between L-serine and L-S-ethylcysteine, and between L-leucine and L-S-ethylcysteine. L-S-Ethylcysteine has a marked inhibitory effect on glycine or serine uptake, but glycine

or serine has relatively little effect on the uptake of L-S-ethylcysteine. Neither L-leucine nor L-S-ethylcysteine seems to compete with each other to any marked extent. These results would indicate the existence of separate carrier systems for L-S-ethylcysteine and the other amino acids investigated.

It may be concluded from these experimental results that a variety of carriers exist in Ehrlich ascites cells that are responsible for concentrative uptakes of the amino acids. This conclusion is supported by results obtained in this Institute showing that DL-valine has little or no effect on glycine uptake by Ehrlich ascites cells (26) but has a large inhibitory effect on DL-methionine uptake (27).

The effects of the metabolic inhibitors, 2,4-dinitrophenol, iodoacetate, and stilbestrol, on amino acid transport indicate that these substances exercise inhibitory effects on transport by their suppression of respiratory or glycolytic energy. It is interesting to note that stilbestrol is a more effective inhibitor of amino acid transport into Ehrlich ascites cells under aerobic conditions than under anaerobic. It is reasonable to account for these results on the hypothesis that the carrier responsible for amino acid uptake is energy-, or ATP- (adenosine triphosphate), dependent and that the carrier breaks down, and is no longer available for amino acid transfer, if the ATP content of the cell is depleted.

Acknowledgments

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THE ACTIVATION OF VARIOUS TYPES OF UTERINE MUSCLE DURING STRETCH-INDUCED CONTRACTION[1]

EDWIN E. DANIEL

With the technical assistance of S. A. RENNER

Abstract

The electrical and mechanical activity of uterine strips from human beings, cats, rats, and rabbits was studied using glass pore surface electrodes and isometric recording of tension. Uterine strips from animals treated with estrogen, or with estrogen and progesterone, were compared with those from pregnant animals. Marked differences in both electrical and mechanical activity were found among the various strips studied. Strips from estrogen or pregnant cats were activated primarily by propagated "all-or-none" action potentials. Propagated action potentials were recorded from rat uterine strips of all types, but they originated from several sites during the same burst, and did not spread over the entire uterus or even a few millimeters in some strips. Mechanical strain appeared to play a role in activation in rat uterus. In strips from uteri of nonpregnant rabbits, action potentials were small, and propagation was slow and limited in extent. Action potential conduction played a minor role in activation in these tissues. Uterine strips from pregnant rabbits resembled those from cats and rats. Action potentials similar to those of animal uteri were recorded from strips of pregnant human uterus. Progesterone pretreatment and pregnancy increased the number of action potentials and duration of contraction. Progesterone pretreatment reduced the sensitivity of cat uterus to stretch. The effect of reduction of external sodium concentration on propagation of uterine action potentials was studied. The results are discussed in relation to the mechanisms underlying electrical activity and to the question of whether electrical activity is a determinant of mechanical activity in the uterus.

Relatively little is known about the electrical activity of uterine muscle. Bozler (1, 2) has found that estrogen treatment alters the cat uterus so that it becomes able to respond to adequate stimulation with an "all-or-none" conducted action potential. Evidence of a similar type of action potential has been presented for the pregnant cat uterus (3, 4). Action potentials were found to be conducted at a rate of 5 to 7.5 cm per second in the estrus and 9 to 12 cm per second in the pregnant uterus (1–4). Studies with intracellular electrodes (4) have shown these uterine action potentials to be spike depolarizations (20–50 mv) showing occasional reversal of the transmembrane potentials (30–50 mv). Similar action potentials have been recorded in pregnant rat (5, 6, 7) and guinea pig (8) uterus using intracellular recording techniques. Conduction has been reported to occur (ca. 4 cm/second) in estrogen-treated rat uterus (9) in studies with external electrodes, but dissociation of individual action potentials in large strips of pregnant rat uterus was observed (7). Progesterone pretreatment has been reported to have little influence on the results obtained with external electrodes (9).

In contrast, Morrison (10) and Rosenblueth (11) found that their studies of nonpregnant rabbit uterus suggested the absence of conducted action potentials. In this laboratory, despite successful recording of action potentials with microelectrodes from cat uterus and from intestinal muscle (4), there has been a

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Contribution from the Department of Pharmacology, University of British Columbia, Vancouver, B.C.

notable lack of success in attempts to obtain similar potentials from rabbit uterus. Because of the technical difficulties associated with microelectrode recordings it was not possible to decide whether this represented a qualitative difference from other uterine muscle.

The technique for recording from the surface of uterine muscle with an external blunt glass pore electrode (4, 12) allows reproducible records of mechanical and electrical activity to be taken for long periods. This method causes no detectable tissue damage. It seemed desirable, therefore, to apply this method to a variety of uterine tissues to answer the following questions:

1. What differences exist between the electrical and mechanical activities of uterine muscle from different species with different hormonal pretreatment and with pregnancy?

2. What is the relationship between electrical and mechanical activity in uterine muscle and how is activation of uterine muscle achieved in vitro?

3. Can the electrical properties of the various uterine tissues provide an explanation for the mechanical properties?

4. In view of the importance of an increased influx of sodium ions in the propagation of electrical activity in other tissues, what is the influence of reduction of the external sodium concentration on the spread of electrical activity in these tissues?

Methods

Animals

Immature rabbits, mature rats, or anestrus cats were prepared by treatment with estrogen or with estrogen and progesterone as previously described (4, 13). Pregnant animals of all species were also studied.

Human Uterus

Human tissues were obtained at Caesarian section. A small portion of tissue adjacent to the incision was taken immediately after delivery and placed in a tube containing Krebs–Ringer bicarbonate solution which had been chilled to 4–6° C. This solution was oxygenated, placed in a refrigerated box, and transported immediately to the laboratory. In most instances about an hour elapsed between the removal of tissue and the rewarming of the tissue for study.

Handling of Tissue

The tissues were removed from the animals and prepared for study as previously described (13, 4, 12). Krebs–Ringer bicarbonate solution, warmed to 35° C and aerated with a mixture of O_2 (95%) and CO_2 (5%) was used as the nutrient medium. Pieces of uterus 3 to 3.5 cm in length were suspended, serous side up, between clamps arranged for recording isometric tension. The whole width of the uterine horn was used except in experiments on tissues from animals in late pregnancy. The latter were divided into strips about 0.5 cm wide. Strips of human gravid uterus were trimmed to similar dimensions and to a thickness of 1–2 mm. A resting tension of 5 g was maintained by adjustment of the distance between the muscle clamps. If 5-g tension failed to initiate rhythmic contractions, increasing amounts of acetylcholine (up to 1 mg) were added to the 35-ml bath and subsequently washed out. In some cases, even

these supramaximal doses of acetylcholine failed to induce contractions.

After spontaneous contractions appeared to be regular, simultaneous recordings of electrical and mechanical activity were made. The differential pore electrode was applied to various areas of the surface of the uterine strip in order to determine whether all or part of the tissue was active and where the activity originated. When sufficient recordings had been made, the recording of mechanical activity was stopped and records taken from two pore electrodes simultaneously. These two electrodes were usually placed parallel to the long axis of the uterine strip and 0.3 to 1.5 cm apart. Recordings from the various portions of the uterus were again taken. Subsequently, the Krebs–Ringer bicarbonate solution was replaced by one containing a 50% mixture of choline–Krebs–Ringer and the process of electrical recording repeated. The nature of the simultaneous records of mechanical and electrical activity in a number of types of uterus under similar circumstances have been reported (12). Finally, a mixture containing 75–80% of choline–Krebs solution was used to replace the 50% mixture, and further records were taken as before. Electrical activity usually began to fail in such solutions and at various stages of this process, the tissue was removed, the endometrium was stripped off, and the myometrium weighed and placed in the oven for subsequent electrolyte analysis. Another segment of the same uterus which had remained in Krebs–Ringer bicarbonate throughout the experiment was usually taken for analysis at this point.

Recording Procedures

Isometric Tension

Isometric tension was recorded by means of an RCA transducer tube No. 5734. The movable anode of this tube was attached directly to one of the muscle clamps by means of a thin strip of phosphor bronze. The other muscle clamp was fixed so that its distance from the first clamp could be adjusted by a screw arrangement. The muscle clamp which was attached to the transducer anode was at the end of the vertical arm of a right angle lever system suspended so as to rotate about the angle. The other, horizontal, arm of the lever system was so arranged that, by moving a known weight along it, the system could be calibrated or preloaded as desired. The output of the mechanical transducer was amplified by a conventional direct-coupled, push–pull system.

Electrical Recording

The pick-up electrodes were glass pore electrodes (14, 15) made from capillary tubing (1–1.5 mm inside diameter) such as was used to make microelectrodes. The ends of this tubing were fire polished until the surface was smooth and the diameter of the opening was reduced to 0.15–0.25 mm. The tubing was filled with the same solution as the muscle bath and inserted on a chemically clean platinum wire .015 in. in diameter so that the end of the platinum wire was about 1 mm from the tip of the electrode. Two such electrodes were used and a Ag–AgCl indifferent electrode was placed in the bath. The two platinum wires were set in a holder which permitted the distance between them to be adjusted and also permitted rotation of the electrodes so that the angle between the line formed by their tips and the surface of the tissue could be made nearly

zero. They were lowered on the serous surface of the uterine segments by means of the coarse adjustment of the microscope base on which the holder was mounted until dimpling of the tissue was observed. They were rotated until dimpling occurred equally under each electrode and raised by means of the fine adjustment until dimpling was no longer visible. By this procedure recordings could be taken reproducibly from the same areas of the uterine surface for many hours. When the electrodes were applied in this way, one of them would not respond to electrical activity occurring only 2–3 mm away at the other electrode. An upwards deflection indicated relative positivity at the recording electrode.

Amplification and Recording

The potential changes between the recording electrode and the indifferent electrode were amplified by means of a direct-coupled push–pull system and recorded along with tension on a 2-channel Sanborn oscillograph. The time constant was 2.5 seconds. The frequency response of the system was limited by the pen recorders and was flat only up to about 30 c.p.s., but was not seriously diminished at 60 c.p.s. Thus the large negative deflections of the action potentials which were found to be complete in 0.020–0.050 second could not have been attenuated or distorted seriously by the pen recorders.

Analytical Procedures

The sodium and potassium and chloride concentrations of dried, digested uterine muscle were analyzed by methods previously described (15, 16, 17).

Interpretations of Records

Records were analyzed according to the following rules:

1. Action potentials smaller than 2 mm (0.1 to 0.05 mv) were not considered in measuring any of the characteristics of these potentials except frequency.
2. The potential difference between the initial positive and the negative peaks was taken to be the amplitude of the action potential. In strips from rat uteri, only those triphasic action potentials which were 75% or more of the maximum amplitude were considered.
3. The measurements of intervals between action potentials were made between positive peaks.
4. In determining the minimal interval between successive action potentials, all measurements were made between successive action potentials of typical configuration and of an amplitude at least 75% of that of the maximum recorded at that electrode during the burst.
5. The action potentials were localized in time relative to tension changes by the position of the peak positive deflection.
6. The slopes of the changes in tension were approximated by dividing them into an arbitrary number of straight lines, starting with that portion of the curve giving maximum slope.

Results

Mechanical Activity

Imposition of a continuous stretch of 5–6 g initiated repetitive spontaneous

contractions in nearly all types of uterus. Strips of uterus from cats treated with estrogen and progesterone were a notable exception. In none of five instances did stretch up to 9–10 g tension initiate contractions. In only one of these instances was acetylcholine, in addition to stretch, sufficient to initiate contractions. Activity was initiated in such strips by elevation of external potassium to 10 meq/liter, indicating that they were capable of response. Progesterone pretreatment also seemed to diminish the responsiveness of some strips of rat and rabbit uterus to stretch, but acetylcholine was sufficient to initiate activity in all of these. Since acetylcholine was required to initiate contractions in some strips from rats and rabbits treated with estrogen alone, no conclusions could be drawn from the limited numbers of strips studied.

The tension changes during a cycle of contraction and relaxation had certain features in common for all uteri except those of the nonpregnant rabbit (see Fig. 1). The rates of tension increase were at first very slow; these rapidly became maximal and thereafter declined progressively as the tension approached its peak. Peak tension persisted for a variable period before the onset of relaxation. Relaxation was the inverse of contraction; though all phases were comparably slower. Only occasionally was the period of increasing tension interrupted by a temporary reversal of the direction of tension change. Intermittent relaxation before the final relaxation occurred more frequently.

Strips from nonpregnant rabbit uteri (Fig. 1) differed from all others in that contracture and relaxation were usually interrupted by partial relaxations and by partial contractions. This pattern of contraction and relaxation was especially marked in tissue from animals pretreated with progesterone.

In all uterine pieces from animals pretreated with progesterone as well as in all pieces from pregnant animals, contractions were more prolonged than those of pieces not exposed to either exogenous or endogenous progesterone. The contractions of uterine pieces from estrogen-treated cats lasted a considerably shorter time than those of any other type of uterine segment (Fig. 1).

Uterine strips from pregnant animals contracted at a maximal rate which was significantly faster than that of any other type of uterine segment. These data are summarized in Table I. The contrast between maximal rates of contraction of segments from a given species was most striking in the case of rabbits, in which strips from pregnant animals contracted about seven times as fast as those from nonpregnant animals. There were differences related to species as well. In particular, the maximal rates of contraction of uterine pieces from nonpregnant rabbits were less than those of comparable strips from cats and rats. Indeed, only in these strips from rabbits was the maximal rate of contraction nearly so small as the maximal rate of relaxation.

Whether or not the differences in mechanical activity are attributable to differences in the manner in which electrical activity spreads over the various types of uterine muscle is of considerable interest. As will be subsequently demonstrated, differences were observed in the electrical activity of the various types of uterine muscle which might explain the slower and more erratic contractions of strips from nonpregnant rabbits as well as some of the other differences noted.

TABLE I

Rates of contraction and relaxation of uterine muscle*

Animal	Condition	No. of animals	Final tension change, g	Maximum rate of tension change (g/sec)	
				Contraction	Relaxation
Cat	Estrogen (E)	4	6.0(22) ±0.5	4.8(22) ±0.4	1.8(12) ±0.2
	Estrogen + progesterone (E+P)	4	9.0(2)	4.3(2)	3.4(1)
	Pregnant	4	7.3(14) ±0.1	11.5(14) ±0.5	2.0(9) ±0.3
Rat	Estrogen (E)	4	5.8(21) ±0.3	1.4(21) ±0.09	0.8(13) ±0.06
	Estrogen + progesterone (E+P)	4	6.0(26) ±0.3	1.8(26) ±0.1	0.5(5) ±0.1
	Pregnant	4	8.1(17) ±0.3	2.4(17) ±0.2	1.1(4) ±0.4
Rabbit	Estrogen (E)	4	2.8(28) ±0.2	0.8(28) ±0.06	0.9(25) ±0.06
	Estrogen + progesterone (E+P)	4	3.6(16) ±0.6	1.0(16) ±0.03	0.7(14) ±0.09
	Pregnant	2	6.2(8) ±0.6	6.5(8) ±0.7	1.3(5) ±0.1

*Variation of means in this and subsequent tables as standard error. The number of values averaged is enclosed in parentheses.

Electrical Activity in Krebs–Ringer Solution

Configuration of Action Potentials

Action potentials of the same configuration were recorded from all uterine strips studied. They were triphasic. The differential electrode became initially positive relative to the indifferent electrode as was to be expected as conducted depolarization approached the electrode. After this upward positive deflection, the potentials shifted rapidly to a negative peak. This negative deflection was invariably the fastest change in potential. The duration of the peak to peak deflection of typical action potentials was 15–30 milliseconds except in strips of nonpregnant rabbit uterus. In these, the duration was 30–60 milliseconds. Following the negative peak, the potential normally returned to the baseline, at a continuously diminishing rate. Variations in this phase were not unusual. The initial positive deflection of these triphasic action potentials was one-fifth to two-fifths the magnitude of the negative deflection.

Variation in action potential configuration during a contraction was minimal in the recordings from pregnant cat uterus (Fig. 2). Action potentials from these tissues were generally similar in amplitude, duration, and configuration at a given recording site and only the frequency changed, decreasing gradually in each burst of action potentials. The electrical activity of some strips of rat uterus was similar. In uterine strips from other rats the action potentials varied in configuration (Fig. 3).

This variation of action potential configuration during a burst did not result from mechanical injury by the electrode, since replacing the electrode at a new

site during a particular phase of the action potential burst did not alter the pattern. Therefore, the variation reflected characteristic changes in the spread of action potentials. The records suggested that each potential spread only over a limited area of muscle. The first action potentials gradually approached but did not pass beyond the muscle electrodes (increasing positive deflections without a negative phase). Subsequently, action potentials appeared to spread beyond the electrodes (acquired a negative phase). Finally, conduction appeared to slow (action potentials prolonged) and then to fail (negative deflections occurring at each electrode independently).

Such variations during action potential bursts were more typical of progesterone-treated uteri than of any other group. A variety of patterns was, however, recorded from the various strips of rat uterus and none was characteristic of its hormonal pretreatment or reproductive status. For purposes of quantitatively comparing the characteristics of electrical activity of rat uterine strips to those from other animals, only the large triphasic spikes from each burst have been considered.

The variation in action potentials of nonpregnant rabbit uterus was also great, but it followed no reproducible pattern (see Fig. 4). At many sites no action potentials were picked up by the electrodes even during contractions, and at others they occurred only occasionally (also see ref. 12).

Amplitude of Action Potentials

Table II summarizes measurements of the amplitude of the peak to peak deflection, minimal interval between action potentials, and conduction rates in the different tissues. The average amplitudes of the action potentials varied considerably from tissue to tissue (Table II). The only entirely consistent difference was the smaller amplitude (less than 0.20 mv) of action potentials recorded from nonpregnant rabbit uterine tissue. In uterine strips from progesterone-treated rats, the maximum voltages[2] were also smaller than in the other uterine strips, with one exception. From all the other uterine tissues, action potentials varying from 0.66 to 1.65 mv and averaging about 1 mv were recorded.

Conduction of Action Potentials

As with other aspects of their electrical and mechanical activity, conduction of electrical activity in strips of pregnant cat uterus was most regular, 8–12.5 cm/second (Table II). There was little variation in conduction velocity of different potentials in a given burst or in conduction of action potentials from different sites over different distances in a given tissue. Variation in conduction velocity of action potentials between similar tissues probably reflected inaccuracies in estimation of interelectrode distance. Regularity of conduction was also noted in estrogen-treated cats but the velocities were slower (3.8–4.4 cm/second). In the one strip in which activity was obtained after proges-

[2]In considering these data, two facts should be kept in mind. First, the values from rat tissues were obtained by excluding all but the large triphasic action potentials in each burst. Second, the values for tissues from nonpregnant rabbits were obtained by ignoring action potentials less than 2 mm in amplitude and by ignoring records which contained no action potentials during contraction. These procedures tend to minimize the differences recorded in Table II.

TABLE II

Action potentials from uterine strips*

Animal	Condition	Amplitude, mv	No. AP's per contraction	Conduction velocity (cm/sec)	Minimum interval (msec)
Cat	E 1	0.67±0.10(10)	2(2)	—	—
	E 2	0.66±0.05(40)	2±1(14)	3.8±0.5(12)	433±33(61)
	E 3	1.17±0.11(32)	6±1(5)	4.4±0.1(8)	633±122(5)
	E 4	1.34±0.34(5)	10(2)	—	—
	E+P 1	1.65±0.07(128)	11±1(8)	5.9±0.1(67)	155+10(14)
	Preg. 1	0.66±0.05(82)	32(2)	12.0±0.3(49)	217±12(4)
	Preg. 2	1.03±0.03(40)	20(2)	9.5±0.2(59)	406±21(4)
	Preg. 3	0.75±0.02(62)	18±1(4)	10.2±0.5(58)	349±15(4)
	Preg. 4	1.31±0.03(176)	15±2(12)	8.8±0.1(87)	508±12(12)
Rat	E 1	0.87±0.14(12)	21±3(10)		345±18(11)
	E 2	1.12±0.12(6)	35±6(5)	7.5†	244±22(6)
	E 3	1.38±0.10(15)	22±2(13)		334±14(15)
	E 4	0.70±0.08(7)	17±5(5)		192±18(6)
	E+P 1	0.68±0.05(9)	19±1(7)		279±19(9)
	E+P 2	0.54±0.21(5)	35±5(4)	10.5†	174(2)
	E+P 3	1.97±0.17(9)	27±1(9)		221±5(9)
	E+P 4	0.33±0.05(6)	30±5(6)		160±12(6)
	Preg. 1	1.02±0.15(6)	54±9(4)		600±40(4)
	Preg. 2	0.75±0.10(4)	31±3(4)	9.0†	294±10(4)
	Preg. 3	0.97(3)	55±5(5)		254(1)
	Preg. 4	1.16±0.09(11)	53±3(14)		160±9(9)
Rabbit	E 1	0.22±0.03(12)	2(2)	1.5±0.4(5)	—
	E 2	0.18±0.02(23)	3±1(6)	3.5(2)	632±76(4)
	E 3	0.10±0.01(21)	2±1(10)	1.8(3)	140(1)
	E+P 1	0.10±0.01(43)	22(2)	—	205±22(4)
	E+P 2	0.12±0.01(133)	56(2)	1.4±0.1(33)	426(3)
	E+P 3	0.14±0.02(28)	30(2)	4.4±0.1(14)	506±28(12)
	E+P 4	0.16±0.02(7)	—	—	—
	Preg. 1	1.09±0.04(55)	44±7(3)	7.7±0.1(38)	261±19(3)
	Preg. 2	0.84±0.12(46)	14±3(3)	7.8±0.4(13)	160(2)
	Preg. 3	1.17±0.18(11)	40(1)	—	404(2)

*E and P refer to treatment with either estrogen or progesterone.
†See Fig. 6.

terone treatment, the conduction velocity was similar, but some action potentials were not conducted.

As with other aspects of electrical activity, velocities of conduction recorded from rat uterine strips were variable. In most cases, this variation followed a recognizable pattern. The first one or two action potentials occurred independently at the two electrodes. Subsequent triphasic action potentials were conducted at rates which varied usually between 6 and 15 cm/second. Then as the amplitude of the action potentials decreased and their positive phase disappeared (Fig. 3) conduction became very slow and in several instances the individual action potentials became dissociated.

In a number of instances, usually in nonpregnant rats treated with progesterone, individual action potentials were dissociated throughout the burst of electrical activity (see Fig. 5(A, B)). This pattern of electrical activity was correlated with the occurrence of smaller action potentials. Usually, despite dissociation of individual action potentials at the two recording sites, the bursts occurred at similar, overlapping times at both recording sites. In extreme

instances, even the bursts of action potentials were dissociated (Fig. 5(C)).

The distribution of conduction rates has been plotted as histograms, displayed in the top row of Fig. 6.[3] This figure indicates that median values in Krebs–Ringer solution (7.5–10.5 cm/second) are similar to the mean values for pregnant cat and rabbit uterine strips.

The strips of uterus from nonpregnant rabbits were most irregular in conduction of action potentials. In strips from estrogen-treated animals it was rare to find any action potentials conducted 0.3 to 1.0 cm and in strips from progesterone-treated animals, conduction was also irregular (Fig. 4). The velocities of conduction were slow for both types of strip, either about 1.5 cm/second or about 4.5 cm/second, suggesting that two modes of spread were being analyzed (Table II).

Minimal Interval between Action Potentials

The minimal intervals between action potentials of maximum or near maximum amplitude have also been recorded and tabulated in Table II. There was considerable variation between the individual strips of a given group. The minimum interval for most types of tissue was between 155 and 217 milliseconds. This interval represents a crude approximation to the relative refractory period of this tissue, since precautions were taken to exclude erroneously small values owing to successive activation of different cells within the field of the electrode (see Methods). It is about the same or slightly less than the duration of the spikes which have been recorded from some of these tissues (3–8). Strips of uterus from estrogen-treated cats and rabbits were exceptions in that the minimal interval was usually over 400 milliseconds.

Action Potential Bursts

With the exception of strips from estrogen-treated cats and rabbits, uterine electrical activity consisted of bursts of more than 10 action potentials. Whenever progesterone had acted upon the myometrium (exogenous or endogenous in pregnancy) the number of action potentials per contraction was increased (Table II), as well as the duration of contraction.

Relation between Contractions and Action Potentials

Simultaneous recordings of electrical and mechanical activity of pieces of uterine muscle were evaluated with one major question in mind. In individual muscle strips, did contractile activity begin, spread, persist, and die out because of the occurrence or absence of preceding propagated electrical activity?

In considering this question, the dependence of mechanical on electrical activity, a difficulty arises. The tension records result from a summation of the active and passive forces exerted by a large number of muscle cells while the action potential records depict the activity of only a limited area. In the light of this difficulty, if the hypothesis of precedence of electrical activity over

[3] In order to avoid inclusion of values for the nonconducted action potentials which sometimes occurred in rat uteri, it was arbitrarily decided to exclude values less than 0.3 cm/second. Values greater than 21 cm/second have not been excluded despite the fact that they probably were obtained when action potentials were initiated between the recording electrodes. They occurred at times when the site of origin of action potentials was shifting as indicated by reversal in the order of precedence of action potentials at the two electrodes.

mechanical is to be maintained, it is necessary (though not proof) to show that action potentials originated in some area(s) of uterine muscle before any increase in tension occurred; that action potentials occurred in most or all of the uterine muscle during or before the period of maximum rate of contraction and before the achievement of maximum tension; that the direction of propagation of electrical activity was everywhere consistent with its origin from the site(s) at which action potentials preceded contraction; and finally that action potentials ceased or decreased in frequency in some areas before the onset of relaxation and ceased in all areas before the completion of relaxation.

Because only two recording channels were available, only a part of this necessary evidence could be obtained. From strips of all types, except those from nonpregnant rabbits, action potentials occurred in most or all areas of the muscle surface during or before the period of maximum increase of tension and before the maximum tension was reached (compare Figs. 7, 8, and 9). From some strips of all types of uterine muscle, except, again, those from nonpregnant rabbits, it was possible to find areas from which action potentials were recorded before any increase in tension could be detected (Fig. 9, cervical). Attempts to localize further the origin of activity suggested that this was under, or very close to, the muscle clamps and therefore inaccessible to further examination.[4] Table III contains examples of the results obtained by analyzing

TABLE III

Strip from:	Electrode at:	Average time to onset maximum rate of contraction* (sec)	Average no. AP's to end of rapid phase	Direction of spread of activity when two electrodes used
Rat-E 3	Tubal end	+6.5	14†	From electrode nearer tubal end toward other electrode; reversed after few AP's
	Middle	−1.6	3	Same
	Cervical end	+0.5	21	Same
Rat, 1 week pregnant 4	Tubal end	+1.45	12	From electrode nearer tubal end toward other electrode; reversed after few AP's
	Middle	+0.55	9	Same
	Cervical end	+0.43	7	Same
Rabbit E 3	Tubal end	0.0 −2.6 −1.2 +3.2 +9.1	††	Variable direction of spread
	Halfway from tubal end to middle	0.0 No. AP's	††	Variable direction of spread
	Middle	+9.8 No. AP's	††	Variable direction of spread
	Cervical	0.0	††	Variable direction of spread

*A positive value indicates that the first action potential preceded onset of the rapid phase of contraction. Figures for rabbit not averaged.
†The total burst was completed; fewer AP's per burst at tubal end.
††Extremely variable, impossible to determine end of rapid phase in some instances; no action potentials until after rapid phase in others.

[4]A small slow increment of tension was almost invariably detectable before rapid contraction. This may have resulted from the onset of activity at the pacemaker.

data from these records and illustrates the failure of uterine strips from nonpregnant rabbits to yield the afore-mentioned necessary evidence. In all uterine strips, action potentials ceased in some and usually in all areas before the beginning of relaxation (Figs. 7, 8, and 9). However, in all uterine strips except those from cats, propagation of action potentials was so variable that it was not possible to determine conclusively that all action potentials were propagated from the site(s) of origin. Data in Table III illustrate the problems. In rat-E3 activity occurred first at the tubal end of the muscle and later on in the middle. However, it began at the cervical end of the muscle earlier than in the middle and more action potentials per contraction occurred at that end than in the remainder of the piece. Apparently action potentials began at the tubal end and spread from there to the middle of the piece. Meanwhile another area in the cervical end had become active before it could have received a propagated impulse. Figure 9 illustrates a similar result. Pregnant rat 4 in Table III illustrates an instance in which activity apparently spread from the tubal end over the remainder of the uterus. However, in both pieces in the table, the direction of propagation was reversed after the first few action potentials and proceeded at least temporarily from cervical to tubal ends. In some instances activity not only appeared to originate from two separate sites, but in addition, these alternated in initiating the contraction as a whole. The only interpretation that could be placed on such results is that a mechanism other than propagation of action potentials initiated contraction in some areas either because propagation failed or because some muscle cells were activated by another stimulus before an action potential could arrive. Quantitative consideration of the data for rat-E3 supports the concept that the individual action potentials fail to spread over the entire strip. Thus, the median rate of propagation of action potentials over short distances in rat uteri was 9 cm/second and the strips used were 3 to 4 cm long. Propagation of a single action potential over the entire strip should have required only 0.33 to 0.44 second, yet 8.1 seconds appear to have been required for propagation of electrical activity from the tubal end to the middle of the strip.

Stretch itself is a stimulus for smooth muscle electrical and mechanical activity. Thus these results in rat uteri could be explained if the stretch which resulted from contraction elsewhere activated other areas so that they in turn initiated electrical activity. Release of a chemical stimulant in response to stretch is also a possible mechanism of activation and might serve as the primary initiation of activity and/or as an intermediary link between stretch and electrical activity. Experiments in which normal electrical and mechanical activity occurred in the presence of large quantities (30 mg/100 ml) of atropine and Dibenzyline suggest that acetylcholine, sympathomimetic amines, and histamine are not involved in co-ordination.

In the nonpregnant rabbit uterus, propagation of action potentials was an entirely inadequate mechanism for co-ordinating contractions since the occurrence of action potentials was irregularly related to contraction (Fig. 8) and since their propagation was slow, uncertain, and limited to short distances (Fig. 4 and Table III). In cat uterus, at the other extreme (Figs. 1 and 7),

there was little to suggest that stretch played any role in activation and coordination of contraction, once it had begun. Pregnant rabbit uterus, like rat uterus, was intermediate in that stretch and propagation of action potentials both seemed to play a role.

In taenia coli (18) it has been demonstrated that action potential frequency is determined by the tension. A similar relation between tension and action potential frequency has been reported for some strips of pregnant rat uterus (7). As illustrated in Figs. 7, 8, and 9 this was not the case for any of the records obtained from the various sites on each of the variety of strips studied. In addition, if uterine strips were stretched by increasing the distance between the muscle clamps, no action potentials occurred despite the increase in tension unless the stretch initiated contraction. The possibility that the magnitude of the passive tension may have preset the initial frequency of action potentials was not tested.

Origin and Cessation of Action Potentials

Direct evidence as to the nature of the mechanisms initiating action potentials was not obtained in this study. There was evidence in all types of uterine strips that cessation of action potentials was preceded by a slowing of their frequency (Figs. 7, 8, 9). In addition, in rat uterine muscle under all hormonal conditions studied, there was evidence of progressive failure of conduction prior to cessation of electrical activity. Figure 3 illustrates the changes, i.e., diminution of the amplitude of action potentials and slowing of conduction with ultimate failure of conduction.

Human Myometrium

A great deal has been written about the in vivo and in vitro electrical activity of the human uterus (19–27). Whether or not many of the slow potential changes at the skin of the abdomen during contraction originate from the uterus is controversial (27). If action potentials play some role in activation of uteri of other species they probably have a similar function in the human uterus. This possibility was investigated by taking tissues from around the incision at Caesarian section and by studying their electrical activity in vitro during spontaneous or drug-induced contractions. There was considerable variation in the amount of electrical activity recorded, but in every instance in which the tension increased rapidly during stretch- or drug-induced contractions (3 out of 7) fast action potentials were recorded (Fig. 10). It was noted that these were all strips of tissue in which a substantial area of serosal surface of the myometrium was not damaged on removal or subsequent trimming. Apparently the way in which myometrium is cut and the number of cells damaged markedly affect the difficulties encountered in attempts to obtain electrical activity from the human uterine muscle.

Effects of Reduction of External Sodium Concentration by Replacement with Choline

The effects of reduction of external sodium on the action potentials and mechanical activity of pregnant cat uterine muscle have been reported (4, 12). The present studies have suggested that the spread of activity over the surface

of the uterus is, in part, by means of self-propagating action potentials. To the extent that these are similar to action potentials in other tissues, the velocity of propagation as well as the amplitude of the action potential should be functions of the rate of depolarization and consequently of the logarithm of the external sodium concentration. In tissues which were sometimes over 2 mm thick, equilibration between the bath and the interstitial sodium takes appreciable time and therefore the effects were analyzed in relation to the time since the bath solution was changed. In the first few seconds the acute effects of the alteration were manifest. Usually this consisted of a prolonged contraction (4, 12) which lasted up to 5 minutes. From 5–15 minutes, equilibration of the new solution with the extracellular space was still occurring. From 15–30 minutes, the equilibration was nearly complete or completed (13). After 30 minutes, there was no reason to think that further change in extracellular ion concentration accounted for changes in uterine activity (13).

Representative data are summarized in Table IV and illustrated in Fig. 11. In uterine strips from cats and rabbits, the data on the conduction velocities and action potential voltages confirm our previous findings (4, 12). In some instances no significant decrease in action potential amplitude or conduction velocity occurred, or it occurred at times unrelated to alterations in interstitial sodium. In other instances, alterations occurred after only 50% reduction in external sodium, which reduces the logarithm of the external sodium concentration only slightly and should have had little effect. The minimal interval between action potentials usually began to increase attendant upon a 50% reduction in external sodium. On changing over to a solution containing 20–25% of the normal sodium concentration, electrical activity became continuous (if this change had not previously occurred) but the interval between individual action potentials became very long, over 1 second in all instances.

In three out of four of the pieces of pregnant rat uterus transferred to a medium containing 50% of the normal sodium concentration, there was a significant diminution of action potential amplitudes and a decrease in conduction velocity. In one case electrical activity stopped entirely. In media containing 20% of the normal sodium concentration a further diminution of mean action potential amplitude occurred and the median conduction rate remained low or diminished further (Fig. 6). In two instances electrical activity disappeared in less than 30 minutes.

In studies on pieces of nonpregnant rat uterus, replacement of 50% of the sodium in the external medium with choline produced a significant decrease in action potential amplitude in only two instances. In two progesterone-treated animals, electrical activity virtually disappeared in this medium. Substitution of choline for 70–80% of the external sodium resulted, however, in reduction in amplitude or disappearance of action potentials within 15 minutes in all but two strips. In these two strips the amplitudes of the action potentials remained as high as or higher than those in Krebs for more than 15 minutes. Median conduction rates were diminished by each reduction in external sodium concentration (Fig. 6). Reduction of external sodium concentration, therefore, affected electrical activity more profoundly in these strips; but, as in the strips

TABLE IV

Effects of reduction in sodium concentration as shown by measurements carried out

Animal	Variable	In Krebs–Ringer	In 50% choline–Ringer for:			In 80% choline–Ringer for:			
			5–15 min	15–30 min	30–60 min	0–5 min	5–15 min	15–30 min	30–60 min
Cat									
Pregnant	1. Conduction (cm/sec)	12.0(49) ±0.3	11.0(11) ±0.7	11.6(14) ±0.7	9.8(15) ±0.3	12.0(10) ±0.3	10.8(15) ±0.7	8.0(9) ±0.3	7.8(35) ±0.1
	2. AP amplitude (mv)	0.66(82) ±0.05		0.70(30)* ±0.04		0.65(12) ±0.08	0.56(18) ±0.03	0.70(18) ±0.01	
	3. Interval (msec)	217(4) ±12		336(6)* ±15		857(2)	2086(2)	2787(6) ±159	
Estrogen	1. Conduction (cm/sec)	3.8(12) ±0.5	3.0(13)* ±0.2						1.6(46) ±0.08
	2. AP amplitude (mv)	0.66(40) ±0.05	0.60(32)* ±0.07						0.65(106) ±0.3
	3. Interval (msec)	433(6) ±33	1228(4)* ±23						1500(3)
Estrogen + progesterone	1. Conduction (cm/sec)	5.9(67) ±0.1	5.5(14)* ±0.1	4.2(32)* ±0.1			4.9(10) ±0.05	3.2(13) ±0.02	
	2. AP amplitude (mv)	1.65(128) ±0.07	2.25(32)* ±0.25	3.06(39)* ±0.15			2.63(52)* ±0.10		
	3. Interval (msec)	155(14) ±10	142(3)*	913(6)* ±16			969(8)* ±44		
Rat									
Pregnant	2. AP amplitude (mv)	0.97(3)		0.50(6)* ±0.04				0.38(6) ±0.03	
	3. Interval (msec)	254(1)		237(6)* ±6				313(6) ±15	
Estrogen	2. AP amplitude (mv)	0.87(12) ±0.14			1.51(10) ±0.16		1.34(8) ±0.10	0.95(18) ±0.13	
	3. Interval (msec)	345(11) ±18			412(10) ±20		443(4) ±8	1323(4) ±99	

TABLE IV (*Concluded*)

Effects of reduction in sodium concentration as shown by measurements carried out (*Concluded*)

Animal	Variable	In Krebs–Ringer	In 50% choline–Ringer for:			In 80% choline–Ringer for:			
			5–15 min	15–30 min	30–60 min	0–5 min	5–15 min	15–30 min	30–60 min
Rat (*concluded*)									
Estrogen + progesterone	2. AP amplitude (mv)	1.97(9) ±0.17		2.52(6) ±0.23	2.45(4) ±0.30		1.63(3) ±0.23		0.57(10) ±0.06
	3. Interval (msec)	221(9) ±5		272(6) ±5	451(4) ±5		1415(3) ±112		1106(10) ±171
Rabbit									
Pregnant	1. Conduction (cm/sec)	7.8(13) ±0.4	8.2(22) ±0.4	8.3(19) ±0.3			7.3(19) ±0.1		
	2. AP amplitude (mv)	0.84(46) ±0.12	2.05(37)* ±0.14				1.17(38) ±0.05		
	3. Interval (msec)	160(2)	496(3)* ±22				680(4) ±16		
Estrogen	1. Conduction (cm/sec)	1.8(3)			1.8(23) ±0.1	1.7(17) ±0.2			1.4(8) ±0.3
	2. AP amplitude (mv)	0.10(21) ±0.01			0.30(63) ±0.02	0.40(38) ±0.03			0.38(33) ±0.03
	3. Interval (msec)	140(1)			2077(6) ±14	1518(4) ±166			2018(4) ±196
Estrogen + progesterone	1. Conduction (cm/sec)	1.4(33) ±0.1		1.4(35) ±0.1				1.2(12)* ±0.1	
	2. AP amplitude (mv)	0.12(133) ±0.01		0.25(89) ±0.01				0.19(22)* ±0.01	
	3. Interval (msec)	426(3)		546(9) ±16				2533(5)* ±128	

*Measurements overlapped two time intervals.

from cats and rabbits, the changes were not consistently related to reduction in interstitial sodium concentration.

The electrolyte contents of these tissues were determined and confirmed our previous findings (4, 12) that large quantities of sodium are bound and that lowering external sodium promotes potassium leakage. The extent of potassium loss did not appear to determine the presence or absence of electrical activity (12).

Discussion

Electrical Activation of Uterine Muscle

The evidence that action potentials initiate and determine the repetitive tension changes induced by continued stretch in cat, rat, and pregnant rabbit uterus is substantial. Action potentials precede rapid contractions and sometimes all demonstrable contraction, and the cessation of action potentials precedes relaxation. In some instances, the possible initiation of action potentials by a small contracture, i.e., contraction without action potentials, could not be excluded because a slow increase in tension occurred before the first recorded action potential. That chemicals can activate uterine muscle in the absence of action potentials has been demonstrated in this laboratory using media containing no sodium and a high potassium concentration (12). It is therefore conceivable that action potentials provide accessory, more rapid activation which is superimposed on a more primitive chemical activating system. Action potentials once initiated spread over the entire muscle strip at a rate sufficiently rapid to activate the entire strip during the rapid phase of contraction. The nature of the electrical record obtained is consistent with the occurrence of the spread of a wave of depolarization by means of local currents in a volume conductor (14, 15). Calculation of the limits of length of the

TABLE V

Activated area

	(1)	(2)*		(3)†	
		(*a*)	(*b*)	(*a*)	(*b*)
Cat					
Pregnant	10.0	.200	.020	2.0	0.2
Estrogen	4.0	.400	.020	1.6	0.08
Estrogen + progesterone	6.0	.160	.020	0.96	0.12
Rat					
Pregnant	9.0	.160	.020	1.44	0.18
Estrogen	7.5	.190	.020	1.43	0.15
Estrogen + progesterone	10.5	.160	.020	1.68	0.21
Rabbit					
Pregnant	8.0	.200	.020	1.60	0.16
Estrogen	1.5	.140	.040	0.21	0.06
				0.49	0.14
Estrogen + progesterone	1.5	.200	.040	0.30	0.06
	3.5			0.70	0.14

NOTE: Rounded off conduction velocities (cm/sec); two values are included where two distinct velocities were sometimes recorded.

*Durations of activation (sec): (*a*) based on the minimum interval between action potentials; (*b*) based on the duration of the peak to peak deflection.

†Lengths of activated area (cm) calculated using different measures of the duration of activation.

activated area (Table V) indicates clearly that large numbers of cells are depolarized simultaneously to produce the effects obtained. In fact, it appears impossible for any record of electrical activity to be obtained under other circumstances (29). Evidence for the existence of a syncytium has not been found (30) and no nerve fibers have been reported to exist in uterine muscle arranged in a manner appropriate to conduction. Decremental conduction cannot be completely excluded in the case of the rabbit and rat uterine strips in which activity from one pacemaker did not spread over the entire uterus. However, no direct evidence of this was found.

Other Mechanisms of Activation of Uterine Muscle

There was no evidence which suggested that the action potentials once initiated were controlled by the tension exerted on the muscle cells. The action potential frequency unlike that for taenia coli (18) bore no relation to tension in any uterine strip. The lack of relation between membrane potential and tension in pregnant cat uterus was demonstrated previously (4). Strips of uterus from nonpregnant rabbits were not activated primarily by propagation of action potentials. Both mechanical and chemical mechanisms are conceivable alternatives. An activating effect of stretch appeared the likeliest possibility. If activation of one portion of muscle was transmitted to other portions by means of the resultant strain on them, the over-all process would be expected to be irregular, nonreproducible, and slow because transmission would depend on the variable sensitivity of the various inactive areas to strain and because the chance that large portions of the uterus would be similarly sensitive is small.

A number of observations suggested that a dual mode of activation also occurs in strips of rat uterus. There were usually at least two sites from which action potentials originated and activation of one usually followed that of the other consistently, suggesting that tension exerted by contraction at one site activated the other. The pattern of action potentials in all these strips suggested a limited spread of each action potential with successive action potentials gradually being displaced along the strip. In most strips propagation of action potentials failed only toward the end of a burst, but in others propagation was absent throughout the burst. The difference between the two types of strip might result from the limited distance of propagation which may be shorter than the interelectrode distance in the latter type of strip. If these patterns of action potential variation result from limited propagation and successive initiation and displacement of action potentials, the processes responsible remain to be elucidated.

Electrical Activity as a Determinant of Mechanical Activity

Whether or not the differences in the rates and patterns of contraction in the various types of strips are explicable in terms of differing participation of propagated action potentials cannot be fully determined until some means is devised to take into account the variation in structure between these uterine strips. However, it is worth noting that there is a parallelism between the speed and completeness of propagation of action potentials and the maximum rate of contraction of various types of uterine muscle. In addition, the longer

duration of contraction in pregnant and progesterone-treated strips of uterus is paralleled by the occurrence of more action potentials per contraction. Finally, the long interval between action potentials in strips from nonpregnant cat uteri could account for the absence of fused contractions in these strips.

The observations clearly indicate that original divergent observations by Bozler (1, 2) and by Rosenblueth (11) as to the mechanism of activation of cat and rabbit uterine muscle were based on real differences in electrical and mechanical activity and emphasize the falsity of assuming a physiological identity between different types of uterine muscle.

The Mechanism Underlying Variation of Uterine Electrical Activity

The quantitative and even the qualitative relationship between transmembrane potential changes and potential changes in the extracellular volume conductor is controversial (14, 15, 31). This is especially so when the external electrode is several hundred times the size of the intracellular one. In the pregnant cat uterus both types of record have been obtained (4, 12) and the extracellular records are explicable in terms of the classical concepts of volume conductor theory (14, 15).

In the case of uterine strips from nonpregnant rabbits, consistent intracellular recordings have not yet been made. The electrical properties of these cells cannot therefore, be compared experimentally with those of cat uteri. If the specific properties of the cell membranes and cytoplasm of these cells are assumed to be similar and if there is no syncytium, it is necessary to postulate differences in cell size or in the geometry of the cellular arrangements in order to explain the observed variation in conduction velocity and action potential size (e.g. between estrogen-treated rat and rabbit uterus). There are, however, no large differences in the diameters or in other dimensions of cells in nonpregnant rabbit, rat, and cat (31 and unpublished observations). Even in pregnant uterine muscle cells, hypertrophy causes an increase in diameter of no more than four times (32). If the conduction velocity is proportional to the square root of fiber diameter (unlikely because of the assumption of a uniform fiber and a uniform resistance per unit length in deriving this relationship (33)), this could account for a difference of twofold. Variation on cell size might account for differences in conduction velocities in cat uterine strips but would not account for all the differences between conduction velocities in nonpregnant and pregnant rabbit uteri nor for the similarities between conduction velocities in pregnant and nonpregnant rat uteri.

If no syncytium exists (30) and electrical conduction is between contiguous cells as the available information indicates in all these types of uterine muscle, then the geometry of cellular arrangements may play an important role in determining the spread of excitation from cell to cell, the potentials recorded in the volume conductor, and the likelihood of conduction failure. However, present knowledge of these cellular arrangements is inadequate. In addition, the possible arrangements are so varied and the mathematics so complex that models do not seem particularly helpful.

So far then, it is clear that important differences in the electrical properties

of different types of uterine muscle occur and are reflected in the accompanying contractions. These differences are influenced by the species and hormonal environment from which the tissues were derived, and the influence is most likely exerted on the geometry of cellular arrangements or on specific properties of the cell membrane and protoplasm.

Progesterone and Pregnancy

Compared to uterine strips from animals which had received estrogen alone, those from animals which had received progesterone as well did not differ in most of the mechanical and electrical properties studied. However, the number of action potentials per contraction and the duration of contraction was always increased by progesterone treatment. The increase in duration of contraction has been noted before (34, 35, 36) in electrically stimulated rabbit uterine strips. There was also suggestive evidence from the cat uterine strips treated with progesterone that contractile activity was more difficult to initiate by stretch. In addition, progesterone may affect conduction since many action potentials were not conducted in strips from cats and rats treated with this hormone.

Strips of pregnant uteri were like progesterone-treated ones in that a longer train of action potentials caused a longer contraction. Presumably this resulted from the action of endogenous progesterone. Increased threshold to stretch or tension and conduction failure in vitro were sometimes noted in strips which had overlaid the placenta. Such studies have been pursued in vivo and will not be dealt with here.

Human Myometrium

The action potentials obtained from human uterus during spontaneous contractions in vitro were similar to those obtained from other types of myometrium. It appears likely that many of the tracings reported previously result from changes unrelated to human uterine electrical activity (27).

Effect of Variation in External Sodium Concentration

The failure of reduction in external sodium to reduce the magnitude of the action potentials recorded from smooth muscle in the external medium in the expected manner agrees with previous findings in this (4, 12) and in other laboratories (37, 38). The necessity of modifying the sodium hypothesis (31) in the light of these results has already been discussed and the possibility of alternative explanations mentioned (4). The data obtained in this study indicate that conduction velocity is not diminished strictly in accord with expectation based on the argument that velocity is a function of the rate of depolarization in the action potential which in turn is proportional to the logarithm of the external sodium concentration (39). These data add further weight to the evidence against the selective involvement of sodium ions in depolarization. The data from this study also indicate that spread of electrical activity in the uterus is ephaptic at least over limited distances. Similar conclusions have been reached for other types of smooth muscle (40–44). Therefore, any alternative explanation of the mechanism of depolarization must provide a mechanism whereby electrical activity in one cell can initiate activity in contiguous ones.

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(For Explanation of Figures see pp. 1347–1348.)

Explanation of Figures

Fig. 1. Tracings of contractions induced by continuous stretch of strips of various uteri studied. These are not idealized diagrams but have been redrawn from more or less typical curves in each instance. Strips from cats at top, from rats middle, from rabbits bottom.

Fig. 2. Electrical activity at two electrodes ½ cm apart on a strip of uterus from a pregnant cat. This record illustrates the regular size and configuration of action potentials in this tissue. Note that positivity of the muscle electrode in this and subsequent figures is signalled by an upward deflection. Conduction velocity was constant at 9 cm/second throughout the burst. Time marks at 1-second intervals at the bottom of the figure.

Fig. 3. (A) Spread of electrical activity in the pregnant rat uterus. This is a record containing many features typical of the electrical activity of strips of pregnant and nonpregnant rat uterus in Krebs–Ringer solution. The electrodes were 0.5 cm apart and parallel to the long axis of the uterus.

Fig. 3. (B) Onset of electrical activity in a strip of uterus from a rat pretreated with estrogen and progesterone. This record illustrates more distinctly than 3A the pattern of electrical activity at the onset of action potential bursts in many rat uterine strips.

Fig. 4. Electrical activity at two electrodes ½ cm apart on a strip of uterus from a rabbit pretreated with estrogen and progesterone. This record illustrates the small size of action potentials from these uterine strips and the failure of many action potentials to be conducted between two adjacent electrodes. Those which appeared to be conducted spread at a velocity of from 1 to 1.5 cm/second. Contrast this figure with Fig. 2. One-second intervals marked at bottom.

Fig. 5. Lack of conduction of individual action potentials in rat uterus. This figure depicts the spread of electrical activity in Krebs–Ringer solution of a horn of the uterus from a rat pretreated with estrogen and progesterone. A: Electrodes 0.4 cm apart in the cervical region. The action potentials do not correspond one to one at the two electrodes and attempts to calculate conduction rates lead to small and variable values. B: Electrodes 0.4 cm apart in the middle of the horn. Note that the activity begins in the top (tubal) electrode in B whereas it began in the bottom (cervical) electrode in A. C: Electrodes 0.4 cm apart in the tubal area of the uterine horn. The activity at the two electrodes was unrelated at the two electrodes not only in the lack of correspondence of individual action potentials but also in the lack of overlap of the bursts of activity.

Fig. 6. Histograms of the distribution of conduction velocities in strips of rat uterus. Left, from pregnant animals; middle, from estrogen treated animals; right, from estrogen- and progesterone-treated animals. The top row of histograms represents the distribution of conduction velocities in 100% Krebs–Ringer solution. The middle row represents the distribution 15 minutes or longer after 50% of the sodium in the medium had been replaced by substitution of choline–Ringer solution. At the bottom are the histograms obtained 15 minutes or longer after 80% (occasionally 75%) of the sodium in the medium had been replaced by substitution of choline–Ringer solution. In all instances, the median conduction velocity diminished as the external sodium concentration was decreased.

Fig. 7. Contractile tension and action potential frequency versus times in a strip of pregnant cat uterus. The reciprocal of the interval since the preceding action potential was taken as the frequency. The first action potential therefore had a meaningless low frequency and was not plotted. Note that maximal tension was approached after the occurrence of only two action potentials counting the first one which was not plotted.

Fig. 8. Tensions and action potential frequencies recorded at the same electrode site a few minutes apart from a strip of uterus from a rabbit pretreated with estrogen and progesterone. Top: Correlation between occurrence of action potentials and over-all increase in tension. During the recording, action potentials accompanied all major increases in tension. At one phase of contraction, they were present, but too small to be analyzed. This degree of correlation was unusual. Action potentials also accompanied one of the three shoulders in the relaxation curve which presumably resulted from localized contraction. Correlation between action potentials and contraction was not precise (see the interval from 24 to 30 seconds). Bottom: Lack of correlation between occurrence of action potentials and over-all increase in tension. In this record, maximal tension was nearly attained before any action potentials were recorded. Thereafter action potentials occurred intermittently five times during intervals of relaxation and once during a slight contraction.

Fig. 9. The relationship between tension changes and action potential frequencies recorded from various parts of a strip of rat uterus pretreated with estrogen and progesterone. In this record, as in Figs. 7 and 8, the first frequency recorded is that of the second action potential.

Top: Electrode on the tubal portion of the horn. The second action potential occurred about 100 milliseconds before the onset of contraction. There were 29 action potentials in all during the burst. The frequency of action potentials varied considerably during the contraction, but had no direct relationship to tension. Middle: Electrode in the middle of the horn. The second action potential in this record occurred when the contraction was already underway, later than in the tubal or cervical portions (see below) of the horn. There were 29 action potentials in this burst as in the one recorded from the tubal portion of the uterine horn. Again, there was no direct relationship between tension and action potential frequencies. Bottom: Electrode on the cervical portion of the uterine horn. The second action potential occurred about 200 milliseconds before the onset of contraction. There were 23 action potentials in the burst, which was shorter than the bursts in the tubal and middle portions of the horn. Subsequent simultaneous recordings with two electrodes indicated that the activity which began in the tubal end of the horn spread about two-thirds of the way to the cervical end. The remainder of the horn was activated by action potentials spreading from the cervical end which were initiated nearly simultaneously with those in the tubal end.

FIG. 10. Electrical and mechanical activity of a strip of human myometrium during a contraction induced by acetylcholine. In this instance, acetylcholine bromide (10 μg) was added to the 35-ml bath. Time marked at 1-second intervals at bottom.

FIG. 11. Effects of replacement of sodium in the external medium by choline on electrical activity of uterine strips of the rat. A. Record in Krebs–Ringer medium. Electrodes 0.3 cm apart on a strip pretreated with estrogen and progesterone. Conduction rate of about 9 cm/second in a number of the action potentials. There were, as usual, a few action potentials which did not appear to be conducted and there was considerable variation in the size and shape of the action potentials. B. Record after more than 15 minutes in 50% choline–Ringer. The action potential bursts became more regular or at least followed the usual pattern. The triphasic action potentials at the left of the record were conducted at a rate of up to 11 cm/second. Conduction became progressively slower and finally failed as the action potentials changed in configuration and decreased in frequency. C. Records after more than 15 minutes in 80% choline–Ringer. Action potentials occurred continuously without periods of prolonged inactivity. The interval between action potentials has become much prolonged, to more than 1 second. Conduction velocity was variable and at the maximum was about 7.8 cm/second. Note the shifting direction of conduction between the third, fourth, and fifth action potentials.

NOTE: Figs. 1–11 follow.

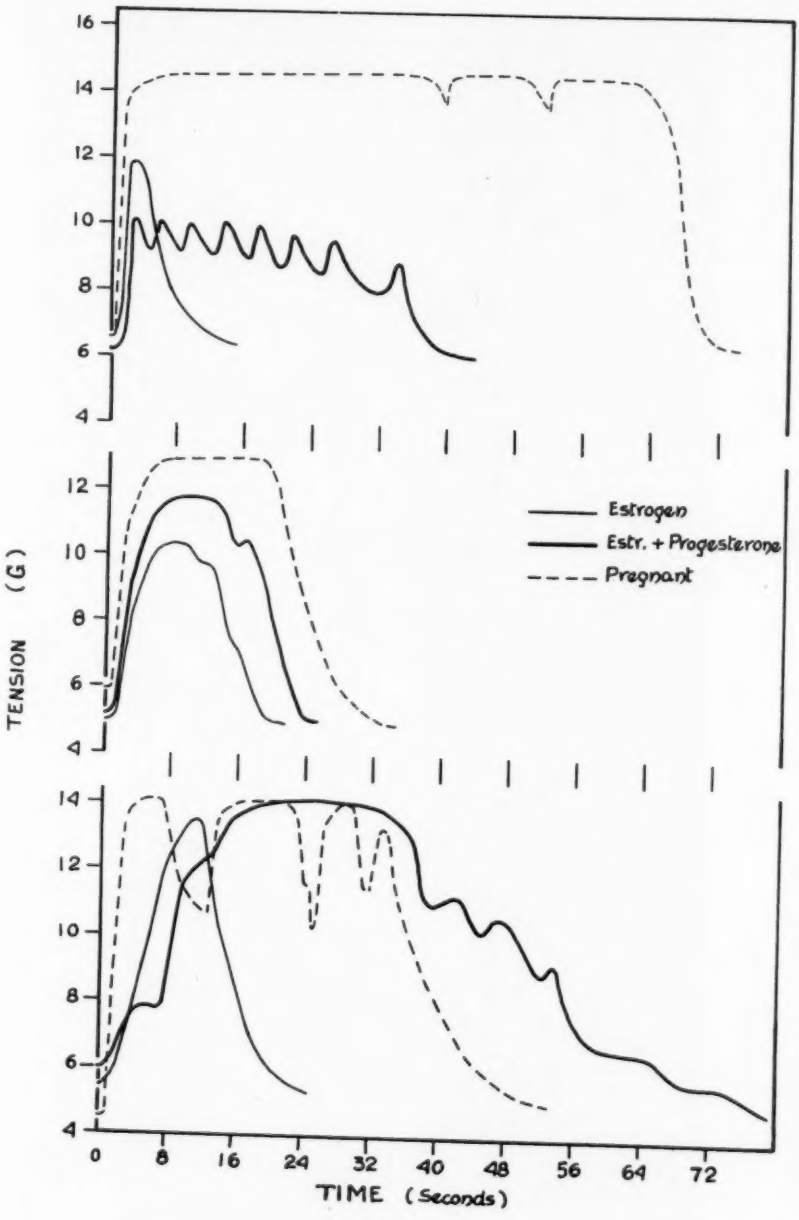
TENSION (G)
TIME (Seconds)
Estrogen
Estr. + Progesterone
Pregnant

FIG. 1.

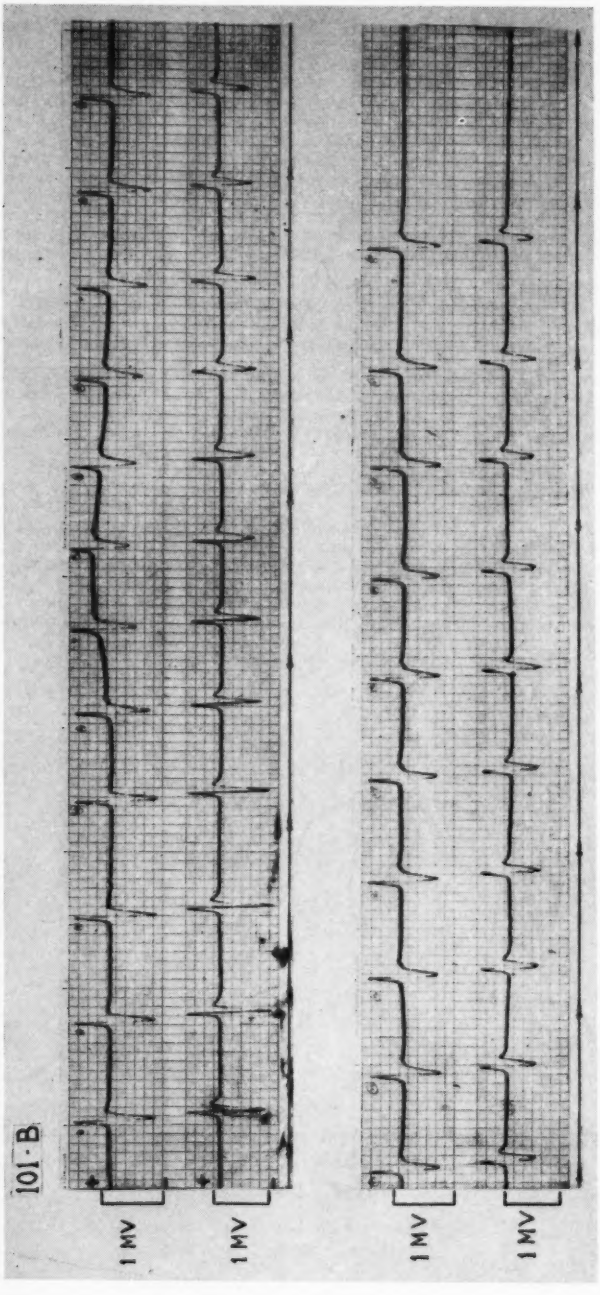

FIG. 2.

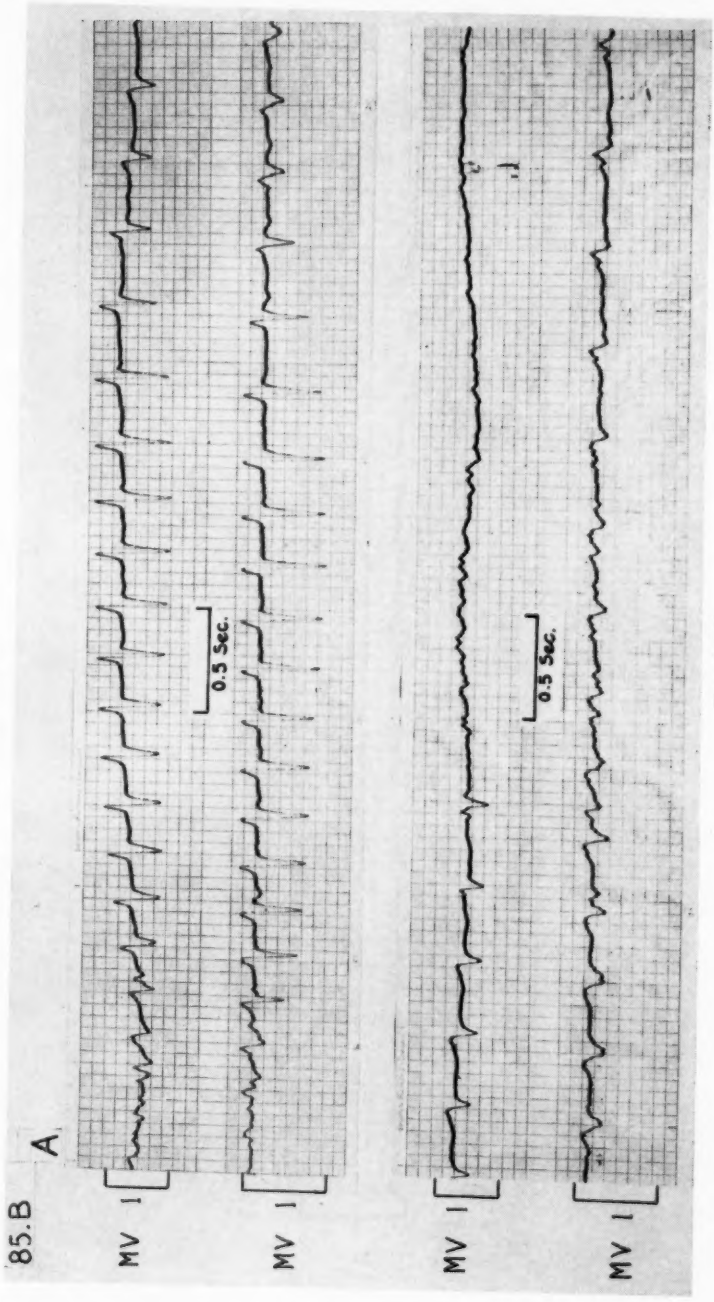

FIG. 3 (A).

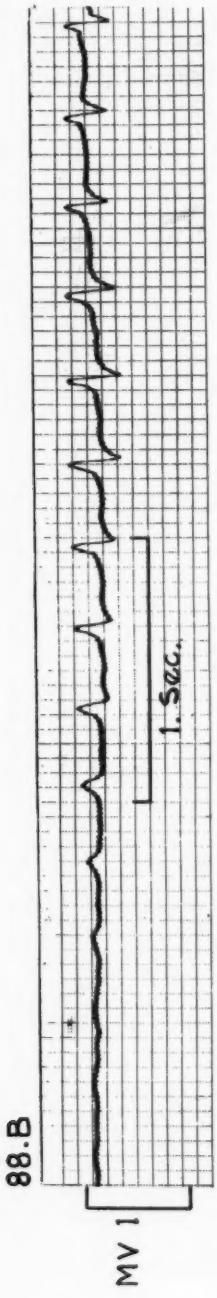

FIG. 3 (B).

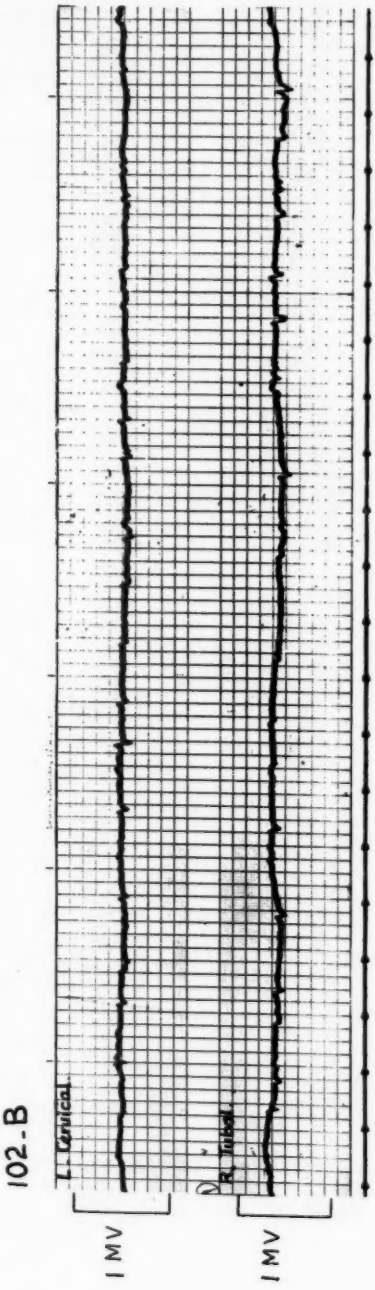

FIG. 4.

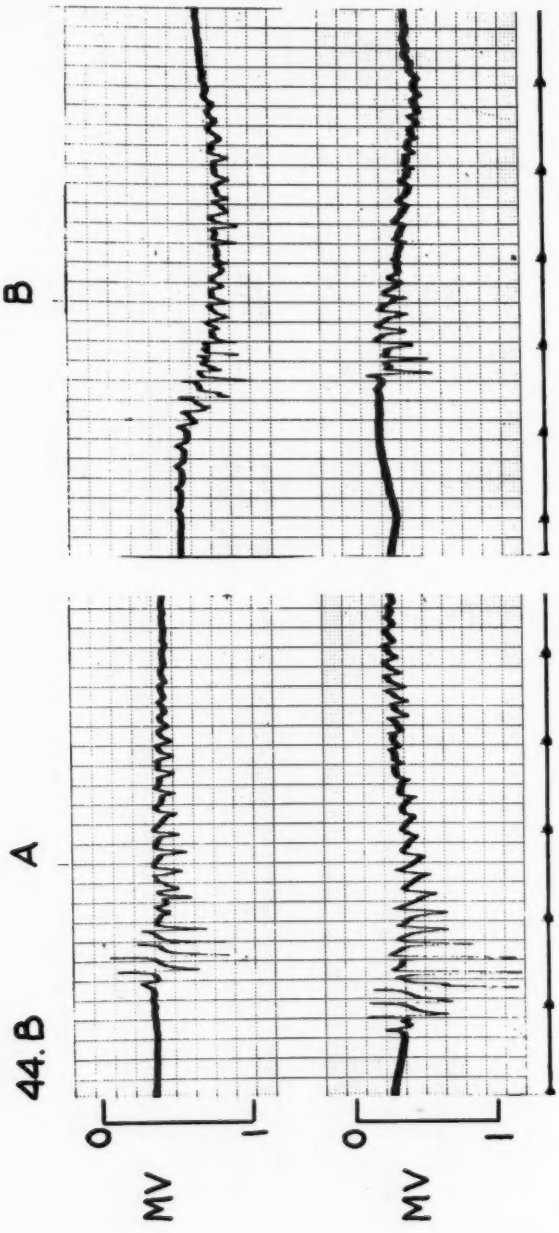

FIG. 5.

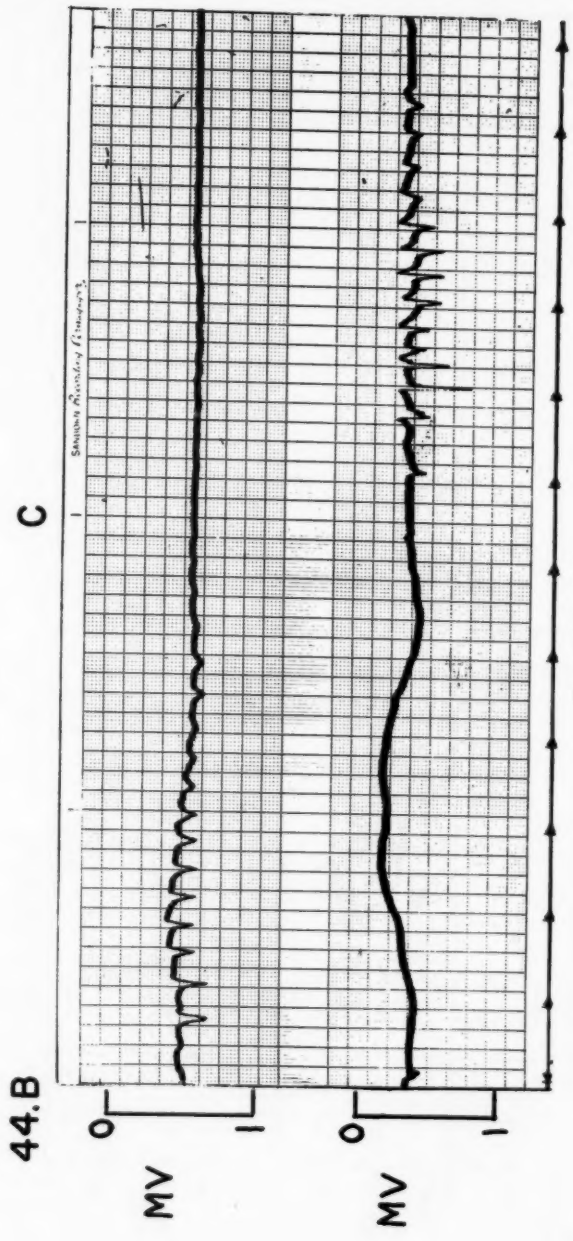

FIG. 5 (*Cont'd.*).

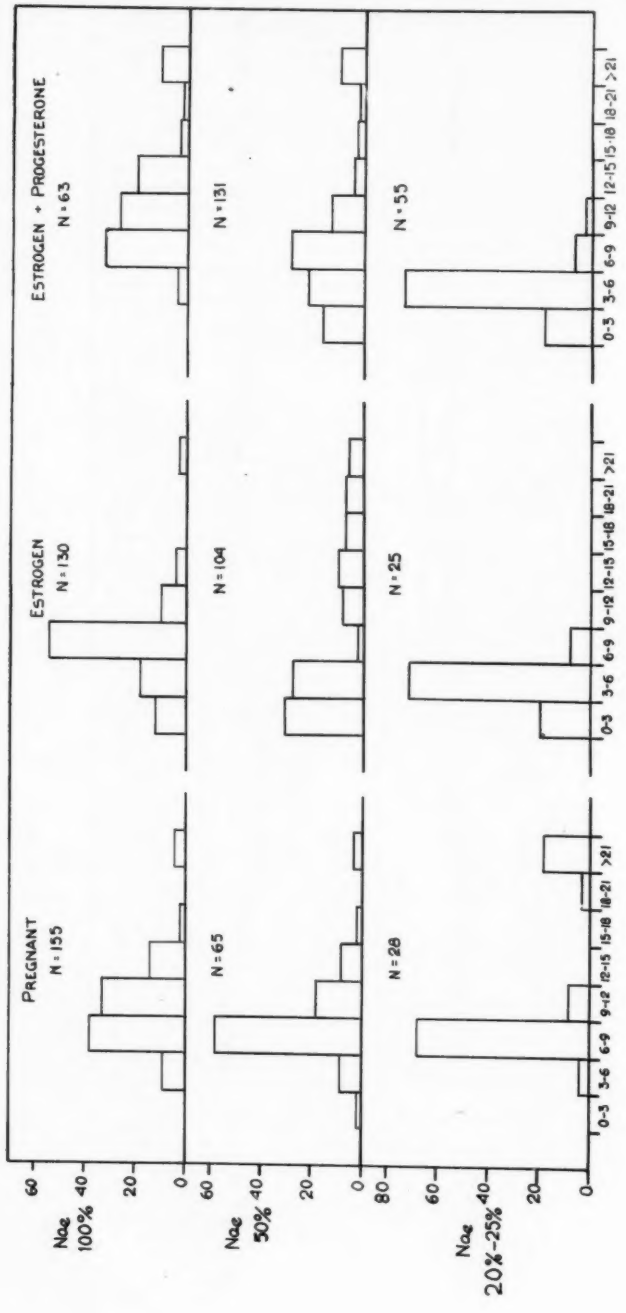
PREGNANT
N = 155
ESTROGEN
N = 130
ESTROGEN + PROGESTERONE
N = 63
N = 65
N = 104
N = 131
N = 28
N = 25
N = 55
Nae 100%
Nae 50%
Nae 20%-25%
0-3
3-6
6-9
9-12
12-15
15-18
18-21
>21
Cm / Sec

FIG. 6.

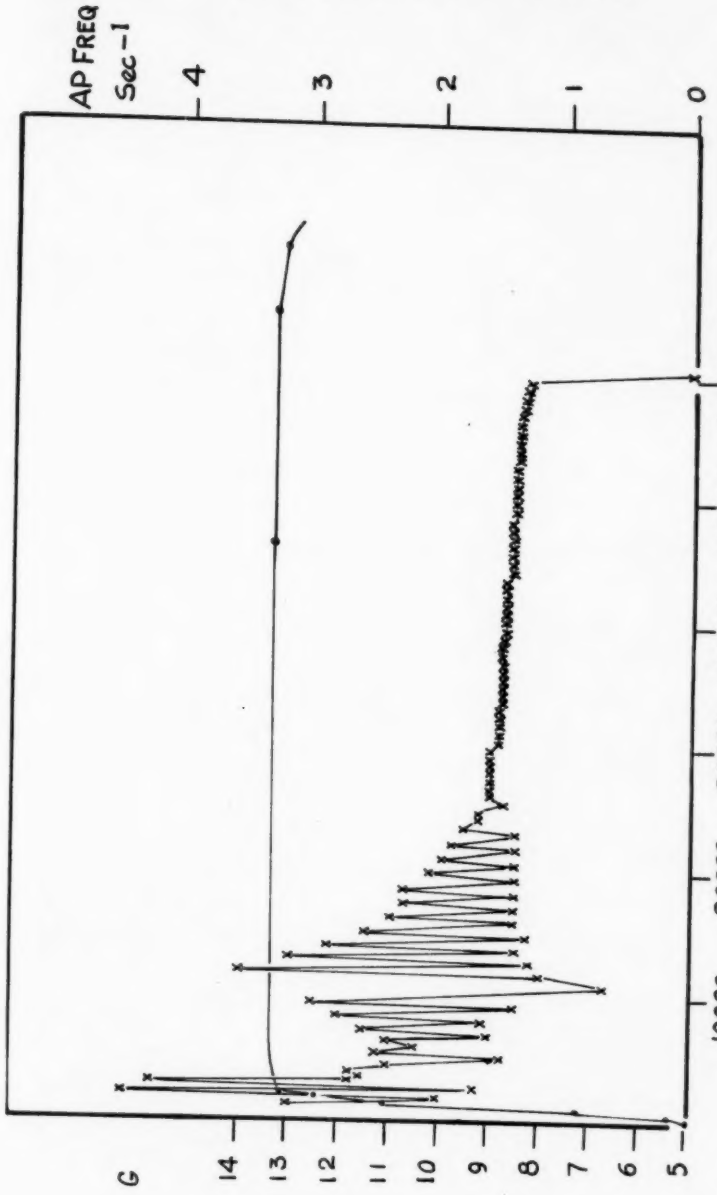
AP FREQ
Sec-1
4
3
2
1
0
G
14
13
12
11
10
9
8
7
6
5
10000
20000
30000
40000
50000
60000
TIME (msec)

FIG. 7.

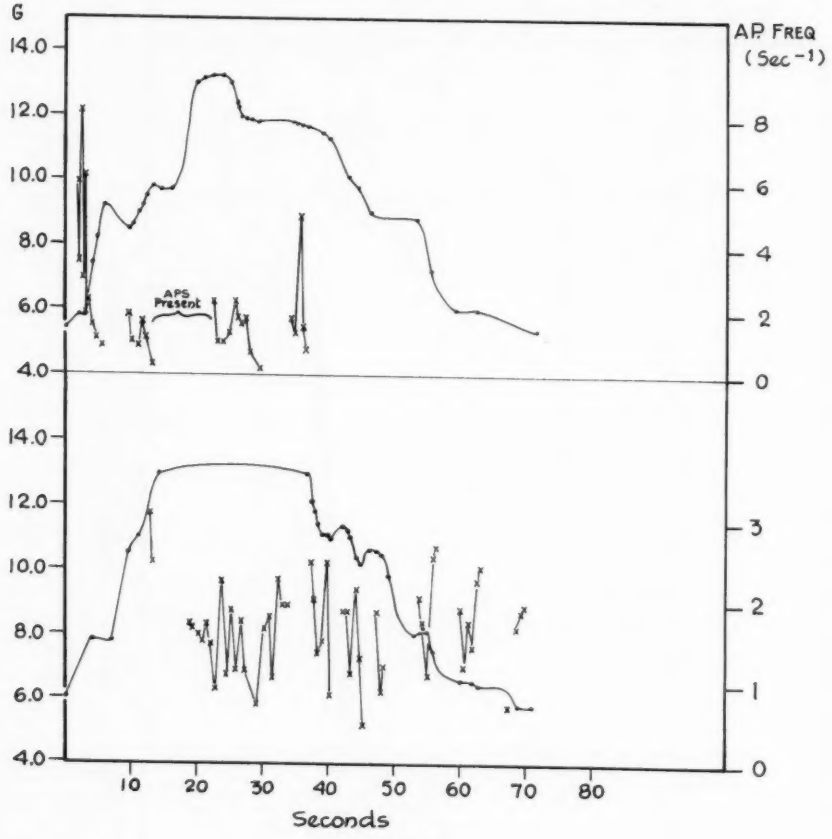
G
A.P. FREQ
(Sec⁻¹)
14.0
12.0
10.0
8.0
6.0
4.0
8
6
4
2
0
APS Present
3
2
1
0
10
20
30
40
50
60
70
80
Seconds

FIG. 8.

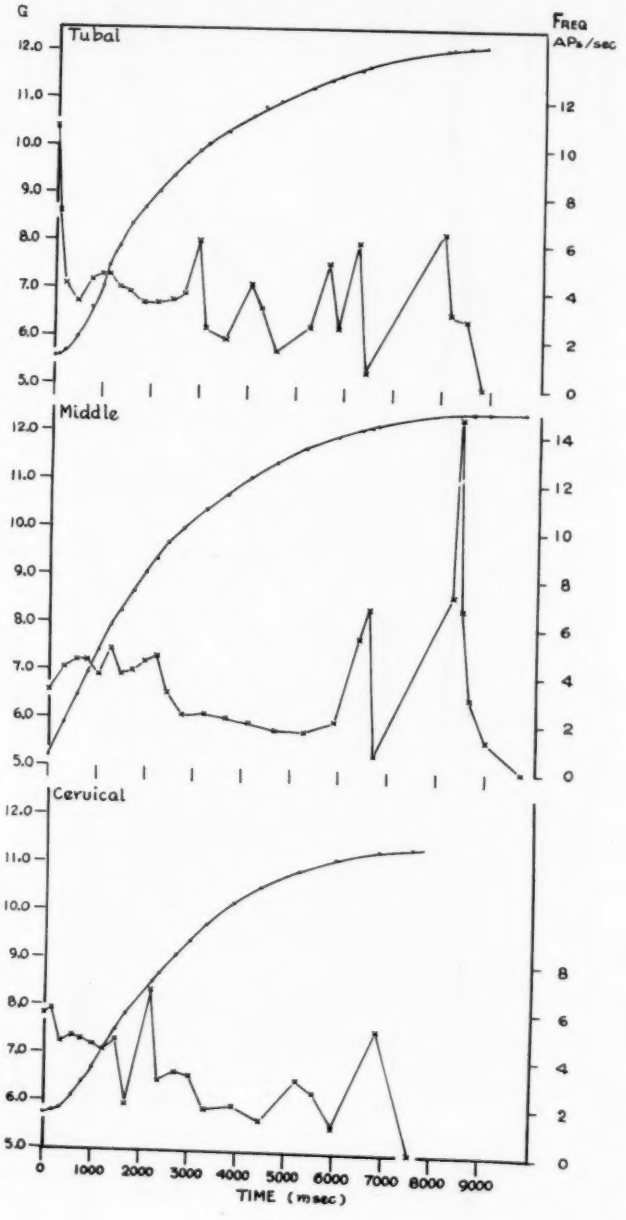
G
FREQ
APs/sec
Tubal
Middle
Cervical
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
14
12
10
8
6
4
2
0
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
TIME (msec)

FIG. 9.

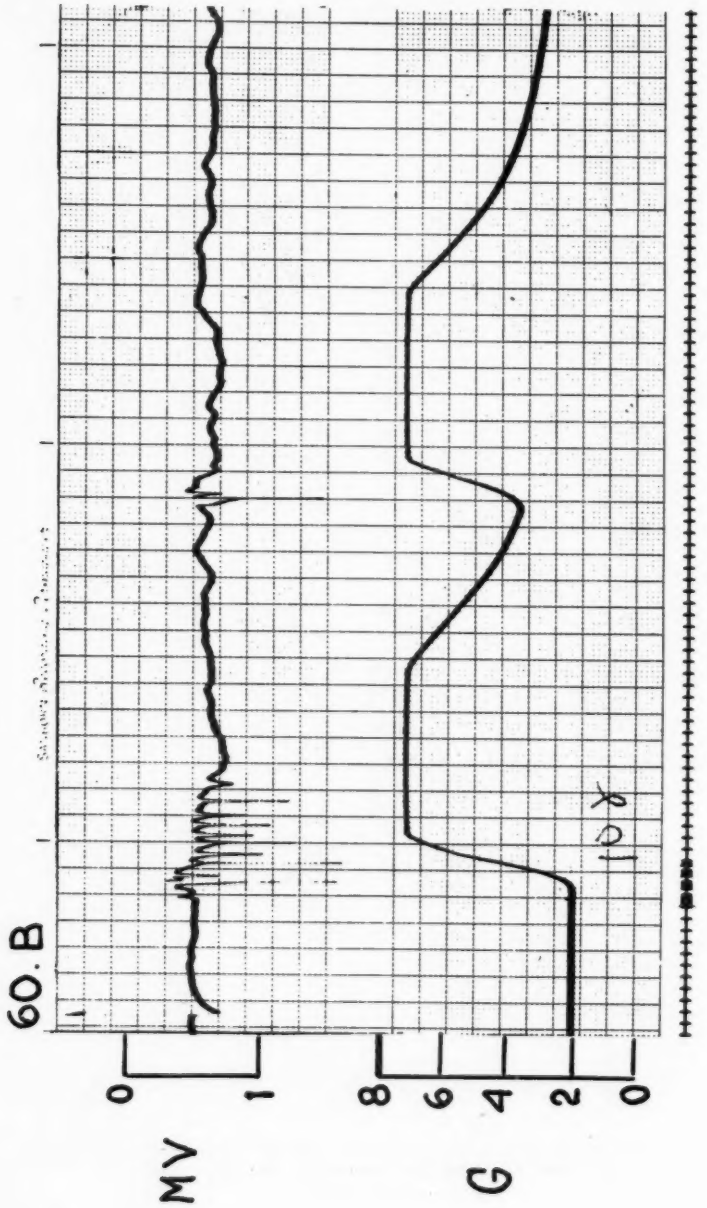

FIG. 10.

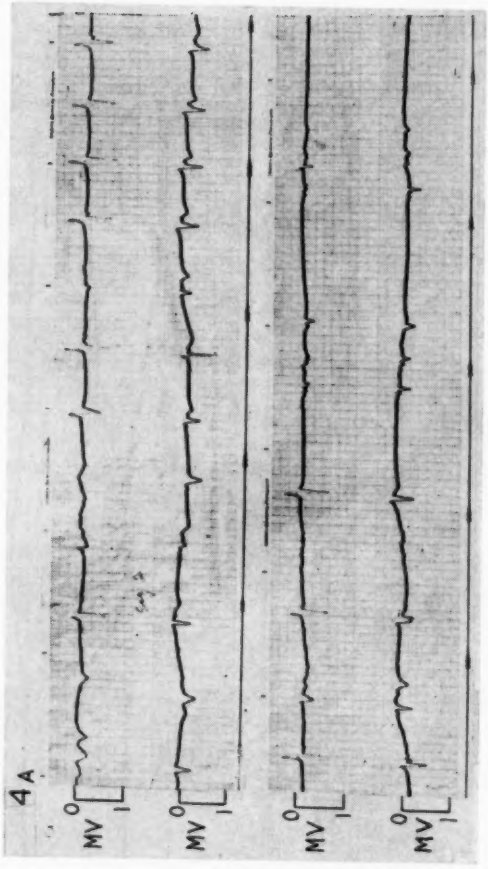

FIG. 11.

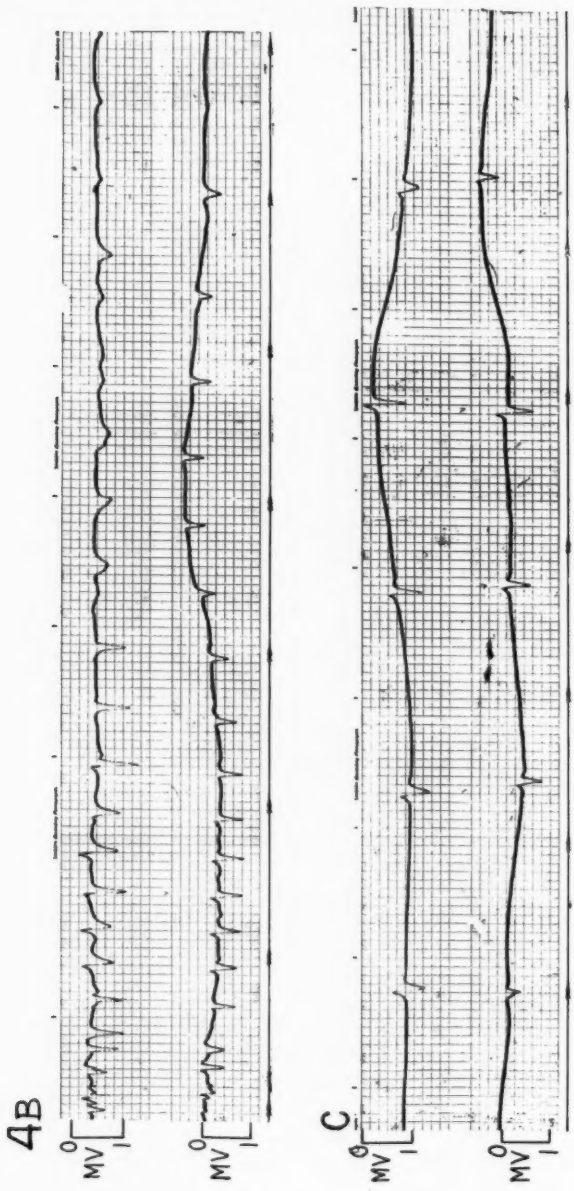

FIG. 11 (*Concl'd.*).

ACETYLCHOLINE IN PERIPLANETA AMERICANA L.

IV. THE SIGNIFICANCE OF ESTERASE INHIBITION IN INTOXICATION, ACETYLCHOLINE LEVELS, AND NERVOUS CONDUCTION[1]

E. H. COLHOUN[2]

Abstract

Inhibition of ali-esterase (ALiE) of roaches by injections of tri-ortho-cresyl phosphate (TOCP) did not result in organophosphorus intoxication. Acetylcholinesterase (AChE) was not inhibited. Topical application of either tetraethylpyrophosphate (TEPP) or O,O-diethyl O-*p*-nitrophenyl phosphorothioate (parathion) to roaches treated with TOCP resulted in poisoning, inhibition of AChE, increases in the amount of acetylcholine (ACh), and disturbances in electrical nervous activity. Similar results were found for roaches treated only with either TEPP or parathion, but prostration was achieved more quickly in roaches first treated with TOCP, suggesting that TOCP treatment potentiated TEPP and parathion poisoning. Studies of electrical nervous activity in roaches showed that nervous conduction was not interfered with by ALiE inhibition. Synaptic nervous transmission was disrupted by inhibition of AChE with TEPP. Axonic nervous transmission was unimpaired although AChE was found to be inhibited.

Introduction

Kearns (1) points out that the mode of action of organophosphorus poisons in insects is not due to inhibition of one enzyme system. The author further stresses that such enzyme systems would be better defined with respect to their function in the normal physiology of the organism. Colhoun (2) has shown that the elements of the cholinergic system in the American cockroach, *Periplaneta americana* L., appeared to be confined to the nervous system. Studies with tetraethyl pyrophosphate (TEPP) have shown a general correlation between acetylcholinesterase (AChE) inhibition, increases in the amount of acetylcholine (ACh), and interference with nervous conduction (2, 3, 4).

The nervous system of the cockroach is known to contain aromatic (5) and ali-esterases (ALiE) (6). Their role in intoxication and nervous function is unknown. Tri-ortho-cresyl phosphate (TOCP) has been used as a selective inhibitor to study the function of ali-esterase in vertebrates (7, 8). Stegwee (9, 10) used TOCP successfully to study the role of esterases in sublethal levels of TEPP poisoning in the housefly. Results reported here for the roach show the significance of AChE and ALiE inhibition in TEPP poisoning and the relative importance of these enzymes in nervous function.

Materials and Methods

Three-week-old male roaches were used for all experiments. They were obtained from laboratory stock reared according to the method of Fisher and Jursic (11).

Treatment of Roaches with Organophosphorus Poisons

TEPP was prepared by Dr. E. Y. Spencer of this laboratory; purity was in

[1]Manuscript received May 9, 1960.
Contribution from Science Service Laboratory, University of Western Ontario Sub-Post Office, London, Ontario.
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accordance with the physical constants given by Hall and Jacobson (12). Solutions of TEPP in acetone were applied topically to the dorsal abdomen. Pure O,O-diethyl O-*p*-nitrophenyl phosphorothioate (parathion), obtained from American Cyanamid Company, New York, was applied in the same manner. The dose of TEPP or parathion was 5 μg per roach.

TOCP was obtained from Eastman Kodak, Rochester, New York. Aldridge (13) has shown that impure TOCP was an inhibitor of AChE. No in vitro inhibition of AChE of the nerve cord of the roach was found at 10^{-2} *M* TOCP, thus showing that TOCP was pure according to the criterion of Aldridge (13). TOCP dissolved in 10 μl propylene glycol was injected into the abdomen of roaches. The dose used was 100 μg per roach, which inhibited ALiE in vivo without inhibiting AChE. This technique was used in preference to that of Stegwee (10), who dissolved TOCP in olive oil, for in the roach greater amounts of TOCP dissolved in olive oil were required to bring about the same inhibition of ALiE found when propylene glycol was used as the solvent. Olive oil probably remained in droplets within the body cavity of the roach. Care was taken with the amount of propylene glycol used for preliminary experiments showed that more than 20 μl was toxic when injected into a roach. Propylene glycol at toxic doses caused immediate convulsions and rapid prostration of roaches; these effects were easily distinguished from those induced by either TOCP or TEPP treatment. At a dose of 10 μl of propylene glycol per roach no inhibition of ALiE or AChE was detected.

Determination of Esterase Activity

AChE and ALiE activity was measured manometrically at 25° C. The main compartment contained 2 ml of $NaHCO_3$ solution, final concentration 0.025 *M*, and 0.5 ml of enzyme preparation. The atmosphere was saturated with 5% CO_2 in nitrogen. For AChE determination the side arm contained 0.2 ml ACh bromide, final concentration 0.01 *M*. The activity of ALiE was determined in Summerson double-arm flasks; 0.2 ml ethyl butyrate dissolved in ethyl alcohol was placed in one arm and the other arm was used for gassing. This avoided loss of ethyl butyrate by evaporation during the gassing operation. The final concentration of ethyl butyrate was 0.01 *M*, which was found to be the optimal substrate concentration for ALiE of the roach. Corrections were made for endogenous acid formation of enzyme preparations in the absence of substrate.

After treatment of roaches with TEPP, care was taken to avoid inhibition of AChE during homogenization of extracted tissue. Nerve tissue was dissected out of the insect and frozen over dry ice. The tissue was homogenized in ice-cold 0.9% saline containing 15 mg of ACh bromide per ml; 0.5 ml of the mixture was placed in the side arm of the Warburg vessel. When the mixture was tipped into the main compartment of the vessel 0.01 *M* ACh bromide was obtained. Until a flask was tipped no hydrolysis of ACh occurred for the concentration of ACh in the side arm inhibited AChE activity. It was unnecessary to take the precaution of preventing in vitro inhibition of ALiE in the manner similar to that used for AChE determinations, for tissues of roaches treated with TOCP were not used until 24 hours after treatment. By combining tissues of

untreated and treated roaches it was determined that free TOCP was not present after this interval.

AChE and ALiE activities were determined in duplicate for each experiment. Four experiments were carried out in each case. The amount of tissue used per experiment and modifications of any of the above techniques are given in the text.

Electrophysiological Studies

Electrical activity of the nerve cord of the roach was recorded with a Tektronix 512 oscilloscope, Tektronix Incorporated, Portland, Oregon, and an Ampex 601 tape recorder, Ampex Corporation, Redwood City, California. To record the electrical activity of the nervous system of the intact roach, small areas of the cuticle were cut away to expose nerve fibers or ganglia, on which were placed chlorided silver electrodes (4). The isolated nerve cord was placed in Ringer's solution (4) and when nervous activity was being recorded part of the nerve cord was suspended upon similar type electrodes.

Extraction of Nerve Tissue and Assay for Acetylcholine

The method and procedure used for the cockroach have been described by Colhoun (4). The amounts of nervous tissue used for each experiment are given in the text. All experiments were carried out in duplicate and each experiment was replicated at least five times. The ACh values reported are given in amounts of ACh bromide; the identity of ACh was confirmed by hydrolysis with AChE and alkali and by the antagonistic effect of atropine.

Results

The Effect of Tri-ortho-cresyl Phosphate Treatment upon Roaches

Treatment of some vertebrates with TOCP results in flaccid paralysis and possible death (14). In part, this is thought to be due to demyelination of nerve tracts. Treatment of roaches with 100 μg TOCP resulted in variable symptoms of lethargy but no other external signs of poisoning. A large number of roaches survived TOCP treatment for 3 weeks at which time the experiment was discontinued. Although relatively normal in appearance at 24 hours roaches treated with TOCP showed internal manifestations of abnormal function. The foregut was often found to contain copius amounts of watery fluid which was regurgitated when the roaches were handled. The mid-gut and hindgut contained undigested food although the roaches were deprived of food during the 24-hour period after treatment. Because the foregut of insects does not absorb water (15), the presence of abnormal amounts of fluid in the foregut of TOCP-treated roaches probably indicates a disturbance in mid-gut function.

Esterase Inhibition in Roaches Treated with TOCP

Twenty-four hours after treatment of roaches with TOCP the enzyme hydrolyzing ethyl butyrate, termed ALiE (16, 10), was found to be completely inhibited in all tissues examined. AChE activity was unimpaired. These results, given in Table I, show that ALiE inhibition was tolerated by the cockroach. Inhibition of ALiE did not result in organophosphorus poisoning in

the roach and was obviously not a factor in tremors and convulsions associated with typical poisoning symptoms (4).

TABLE I

Ali-esterase and acetylcholinesterase activity (mg/g/hr) in tissues of the roach at 24 hours after treatment with 100 μg TOCP per roach

Tissue	Untreated roaches		TOCP-treated roaches	
	Q_{ALiE}*	Q_{AChE}†	Q_{ALiE}	Q_{AChE}
Thoracic nerve cord	20.32±4.1‡	222.74±18.5	0	217.52±15.6
Sixth abdominal ganglion	16.00±3.1	381.62±19.3	0	375.66±12.7
Coxal muscle	1.78±0.8	—	0	—
Fat body	11.36±2.3	—	1.38±0.8	—
Whole gut	8.18±2.1	—	0	—
Dorsal abdominal cuticle	12.04±4.9	—	0	—

*0.01 *M* ethyl butyrate.
†0.01 *M* ACh bromide.
‡Standard deviation.

TABLE II

Ali-esterase and acetylcholinesterase activity (mg/g/hr) in tissues of the roach at 10 days after treatment with TOCP

Tissue	Normal roaches		TOCP-treated roaches	
	Q_{ALiE}*	Q_{AChE}†	Q_{ALiE}	Q_{AChE}
Thoracic nerve cord	22.66±3.6‡	230.56±18.1	10.24±1.8	225.80±15.4
Fat body	10.05±2.5	—	10.14±2.6	—
Whole gut	8.94±1.9	—	9.26±1.3	—

*0.01 *M* ethyl butyrate.
†0.01 *M* ACh bromide.
‡Standard deviation.

TABLE III

Acetylcholinesterase activity (mg/g/hr) in tissues of dead roaches at 24 hours after treatment with 100 μg TOCP per roach

Tissue	Normal roaches, Q_{AChE}*	TOCP-treated roaches, Q_{AChE}
Thoracic nerve cord	222.79±16.4†	218.06±21.8
Sixth abdominal ganglion	356.63±12.2	362.85±19.3
Fifth leg nerve	155.02±24.9	167.55±30.6

*0.01 *M* ACh bromide.
†Standard deviation.

Some 10 days after TOCP treatment, a number of tissues were examined for ALiE activity. As shown in Table II, ALiE, known to be inhibited at 24 hours (Table I), showed a high amount of activity at 10 days. TOCP treatment of roaches did not result in lasting inhibition of ALiE. Therefore it is not known whether roaches would indefinitely survive complete inhibition of ALiE.

Occasionally a number of roaches died after treatment with TOCP and although they died in a manner distinct from that found with TEPP poisoning, the possibility existed that AChE was inhibited in the dead roaches. The data

given in Table III show that AChE was uninhibited. The cause of death is unknown. It is doubtful whether death was directly due to ALiE inhibition, for the number of insects found to die after TOCP treatment was less than 10% of the total number of roaches treated. Furthermore, a doubling of the TOCP concentration did not cause a significant increase in mortality.

The distribution of ALiE in tissues of the roach is shown in Table I. It is at once evident that although the highest ALiE activity was found in the thoracic nerve cord the enzyme was present in non-conductive tissue. This distribution is different from that of AChE in the roach, where Colhoun (2) showed that AChE activity appeared to be limited to nervous tissue. The non-specific distribution of ALiE in roach tissues together with lack of toxicity to the roach when ALiE was inhibited is a hint that ALiE in the nervous system is not intimately associated with the mechanism of nerve transmission. Further evidence of this suggestion is found in the histochemical data of Connel (6), who showed that ALiE in the thoracic nerve cord of the roach was found only in interganglionic and thoracic nerve fibers, in a few localities in the synaptic areas and centrally between the interganglionic fibers. In sharp contrast, AChE was found along the length of nerve fibers and concentrated towards nerve endings in ganglia; the neuropile, containing a large number of synapses, showed a heavy concentration of AChE.

The inability of the ALiE of the roach to hydrolyze ACh is shown in Table I, for the amount of hydrolysis of ACh by tissue homogenate in which ALiE was inhibited was about the same as that found where ALiE was uninhibited. This result is in agreement with the statement of Augustinsson (17) that ali-esterases do not hydrolyze choline esters.

It has been said that TOCP is converted to an active inhibitor of AChE in mammals (13). No evidence of conversion has been found in the roach, for as shown in Table I AChE was not inhibited in nerve cord at 24 hours after treatment with TOCP. Moreover, the AChE of homogenates of the thoracic nerve cord of the roach was not inhibited after 1-hour incubation with 10^{-2} *M* TOCP. In contrast the I_{50} of ALiE was 10^{-5} *M* TOCP.

The Effect of TEPP Treatment upon the AChE Activity and ACh Content of the Nerve Cords of Roaches First Treated with TOCP

The results so far have established that ALiE inhibition appears to be of no significance in organophosphorus poisoning in the roach. Stegwee (10) showed that TEPP treatment of houseflies inhibited both AChE and ALiE, but that houseflies first treated with TOCP were less sensitive to a dose of TEPP than were flies treated with TEPP alone. Experiments were performed to test the importance of ALiE in roaches treated with TEPP. The results given in Table III show that roaches were more sensitive to 5 μg TEPP when first treated with 100 μg TOCP. Clearly a difference has been found between the housefly and roach, for roaches first treated with TOCP for 24 hours and then treated with a lethal dose of TEPP became prostrate in about one-third of the time required to bring about prostration in roaches treated with TEPP alone. TOCP appeared to potentiate TEPP poisoning. This effect was also found

when 5 μg of parathion was used instead of TEPP. The time to prostration of TOCP and parathion-treated roaches was 112 ± 28* minutes compared with 201 ± 65 minutes for roaches treated with parathion alone. DuBois *et al.* (8) found that TOCP potentiated malathion poisoning in the rat. They stated that the increased toxicity of malathion was probably due to inhibition by TOCP of enzymes responsible for the detoxication of malathion. In like manner it could be suggested that TOCP inhibits enzymes, among them ALiE, in tissues of the roach and so permits topically applied TEPP to reach the AChE of the thoracic nerve cord more quickly than in roaches treated with TEPP alone. This possibility will be more fully understood if it is shown that tissues of the cockroach contain enzymes hydrolyzing TEPP (TEPP-ase) or whether a concentration effect of TEPP is brought about by TOCP inhibiting a number of unspecific enzymes able to be phosphorylated by TEPP.

The more rapid inhibition of AChE by TEPP in the thoracic nerve cord of TOCP-treated roaches is shown in Table IV. TEPP was applied topically to the dorsal abdomen. Its passage to the thoracic nerve cord entailed trans-

TABLE IV

Acetylcholinesterase activity and acetylcholine content of thoracic nerve cords of roaches injected with TOCP* and topically treated with TEPP† at 24 hours

Time after treatment with TEPP	AChE activity (%)		μg of ACh in eight thoracic nerve cords	
	TOCP and TEPP	TEPP	TOCP and TEPP	TEPP
0	100	100	2.47 ± 0.03	2.47 ± 0.04
22.5 ± 6.3‡ min	25.7 (22.1–30.6) prostrate	64.9 (55.7–73.7)	(*a*) 2.67 ± 0.15 (*b*) 2.49 ± 0.06	2.51 ± 0.13
62.5 ± 22.4 min	0	20.5 (15.8–22.3) prostrate	2.39 ± 0.06	2.90 ± 0.03
100 min	0	0	—	—
6 hours	0	0	(*c*) 2.48 ± 0.18 (*d*) 3.20 ± 0.23	2.35 ± 0.11
24 hours	0	6.9	4.29 ± 0.29	4.56 ± 0.31

*100 μg TOCP per roach.
†5 μg TEPP per roach:
(*a*) short duration hyperactive symptoms after TEPP treatment,
(*b*) lethargic symptoms after TEPP treatment,
(*c*) as in (*a*),
(*d*) as in (*b*).
‡Standard deviation.

location through tissues or blood. At 22.5 minutes nearly three times as much AChE was inhibited in the nerve cords of "TOCP- and TEPP-treated" roaches as in TEPP-treated roaches, which require 62.5 minutes to reach this level of AChE inhibition. Significantly, prostration resulted, after either treatment, at the same degree of AChE inhibition. Fernando *et al.* (18) used topically applied radioactive TEPP and parathion to determine tissue uptake within the cockroach. The highest amount of these substances was found in the foregut and only a small amount was recovered from the nerve cord. Since TOCP was thought to interfere with gut function, it is possible that malfunction of the alimentary tract permitted less uptake of TEPP by gut from blood and hence a

*Standard deviation.

greater concentration of TEPP was available to inhibit the AChE of the nerve cord.

The ACh content of the thoracic nerve cord of TOCP- and TEPP-treated roaches was assayed for comparison with amounts of ACh known to increase after TEPP treatment alone (4). For ACh determinations the thoracic nerve cord was chosen as locomotory in-co-ordination, and hyperexcitability induced by TEPP treatment appeared to originate in this part of the central nervous system. The results given in Table IV show that the inhibition of ALiE by TOCP treatment had no effect upon the ACh content of the nerve cord, a result different from that obtained by Stegwee (10), who found a slightly lower amount of ACh in flies treated with TOCP. At prostration, after treatment with TEPP, the ACh content of the thoracic nerve cord of TOCP-treated roaches became slightly elevated by an amount about half that found in roaches treated with TEPP alone. The reason for this smaller increase in ACh may be found in the observation that when TOCP-injected roaches were topically treated with TEPP they did not exhibit to the same degree the hyperexcitability of roaches treated with TEPP alone. TOCP- and TEPP-treated roaches which showed some excitability were divided from those which became prostrate in a lethargic manner. Table IV reveals an ACh increase only in the nerve cord of the hyperactive roaches. The reasons for the different response are unknown; it may be the result of faster and greater penetration to the nerve cord or to an effect of TOCP not yet evaluated. However, in general, a correlation has been obtained between the rate of prostration of roaches, the inhibition of AChE, and increases in the amount of ACh. AChE inhibition has been demonstrated to be of importance in TEPP poisoning in the roach. Some 6 hours after prostration the ACh content of thoracic nerve cord of roaches treated with either TOCP and TEPP or TEPP alone did not increase, a finding previously reported by Colhoun (4) for TEPP poisoning. No precise explanation has been found of why the ACh content should not increase above that found earlier at prostration though it may leak into the blood (3, 4). If the in vivo electrical nervous activity of the roach ceases at prostration, then a more adequate explanation would be found. Accordingly experiments were carried out to determine the relation of nervous activity to ACh and to AChE inhibition.

Electrical Nervous Activity in Roaches Treated with TOCP and TEPP

The results so far recorded show that inhibition of ALiE by TOCP does not result in intoxication or in disturbances in the amount of ACh. The data given in Table V show that the gross electrical activity of intact or isolated nerve cords of TOCP-treated roaches was comparable to that of untreated roaches, first evidence that ALiE is not essential to the physiological mechanism of nerve transmission in the roach.

Experiments were then carried out to test the role of AChE inhibition in nervous function. Both normal roaches and those treated with TOCP were topically treated with 5 μg TEPP. Electrical activity was recorded from the nervous system of intact treated roaches and from nerve cords isolated from roaches so treated. The results given in Table VI show that the nervous activity

TABLE V

Electrical nervous activity of nervous tissue of the roach at 24 hours after treatment with TOCP*

Nervous tissue region	Untreated roaches		TOCP-treated roaches	
	Intact roach	Isolated nerve cord	Intact roach	Isolated nerve cord
Spontaneous activity recorded between third and fourth abdominal ganglia	+	+	+	+
Cercal nerve – giant fiber synapse†	+	—	+	—
Fifth leg nerve motor activity afferent activity	+	—	+	—

NOTE: + = normal pattern of nerve activity.
*100 μg TOCP per roach.
†Observed in the abdominal nerve cord postsynaptically to the sixth abdominal ganglion.

TABLE VI

Effect of topical treatment with TEPP* upon the electrical nervous activity recorded in the abdominal nerve cord of normal and TOCP-treated roaches†

Time after TEPP treatment (minutes)	Normal roaches and TEPP		TOCP-treated roaches and TEPP	
	Intact nerve cord electrical activity	Isolated nerve cord electrical activity	Intact nerve cord electrical activity	Isolated nerve cord electrical activity
0	+	+	+	+
20	+-++	+	++	0-+
65	++	0	+-++	0

NOTE: 0 Block to some very small nerve fiber activity.
+ Normal nervous activity.
++ High pattern of nervous activity consisting of high frequency volleys of nerve impulses from different nerve fibers.
*5 μg per roach at 24 hours after TOCP treatment.
†100 μg per roach.

of roaches treated with TOCP and TEPP became abnormal before that of roaches treated with TEPP, a finding agreeing with evidence in Table IV for the rate of inhibition of AChE and increases in the amount of ACh. When electrical nervous activity was recorded from the abdominal nerve cord of an intact treated roach, from the time of treatment to prostration, evidence of abnormality was discerned as prolonged volleys of mixed nerve impulses not observed in an untreated roach. At prostration the volleys of nerve impulses increased considerably over those observed shortly after treatment. No difference was to be seen between roaches treated with TOCP and TEPP and those treated with TEPP alone, when the time to prostration was taken into consideration. Since AChE was not completely inhibited at prostration, block of nervous activity was not expected. Relative to progressive inhibition of AChE the observed volleys of nerve impulses appeared to be evidence of facilitation; this was most intense when ACh was detected to increase. Nerve cords were removed from treated roaches at prostration. The level of nervous activity was considerably depressed below that recorded for nerve cords in intact roaches; in many instances nervous activity was less than that found with normal isolated nerve cords. In comparison with results for nerve cords of

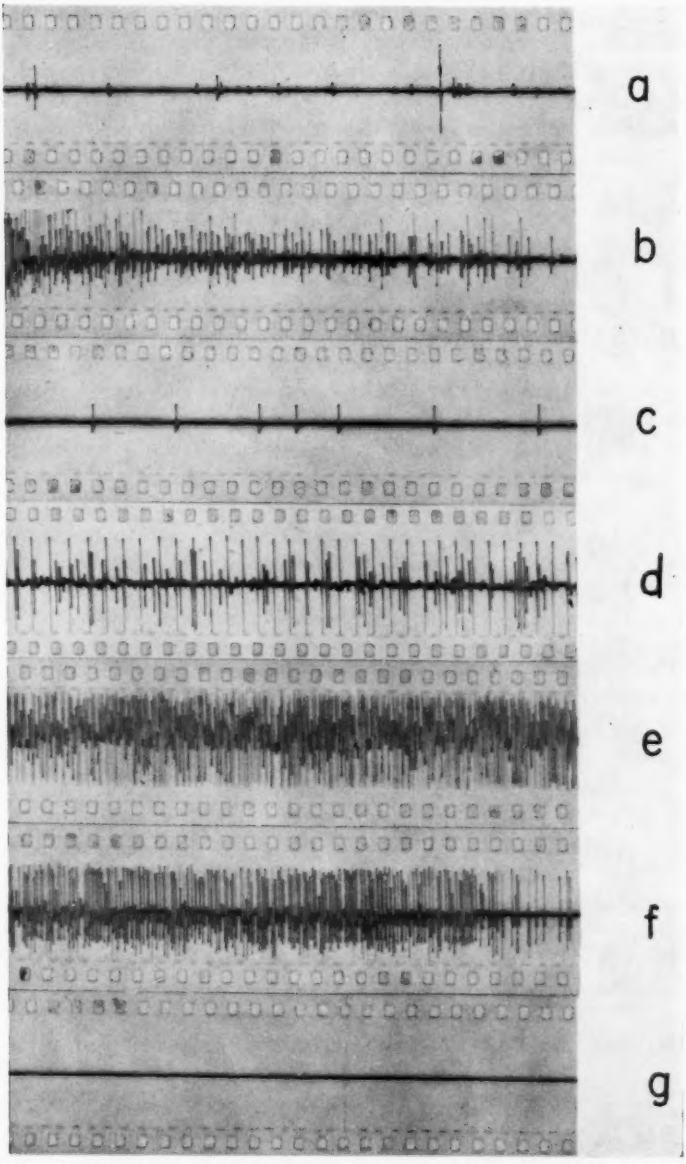

FIG. 1. Electrical nervous activity recorded between the fifth and sixth abdominal ganglion of intact normal or TEPP-treated roaches. (*a*) Normal spontaneous activity; (*b*) normal sixth abdominal postsynaptic response to air puff stimulation of the anal cerci; (*c*, *d*) spontaneous patterns of nervous activity at 6 hours after TEPP treatment; (*e*) high frequency postsynaptic sixth abdominal discharge in roach giving (*c*, *d*) following air puff stimulation of anal cerci; (*f*) spontaneous after discharge resulting from (*e*); (*g*) block after (*f*).

intact roaches the electrical nervous activity of isolated nerve cords appeared blocked, thus depending upon the type of preparation used for recording nervous activity, distinct conclusions could be arrived at with respect to correlations with ACh and AChE. Obliteration of nervous activity found to occur in vivo by isolation of a nerve cord appears to be a result of elimination of afferent nervous pathways.

Electrical Nervous Activity in Nervous Tissues of Roaches at 6 Hours after Treatment with TEPP

Colhoun (4) previously pointed out that correlations between ACh increases, AChE inhibition, and nervous activity were not apparent 6 hours after topical treatment of roaches with TEPP. As shown in Table IV this result has been confirmed. No evidence is available of the in vivo nervous activity of roaches prostrate for some time after TEPP poisoning. This evidence is essential to a proper understanding of the mode of action of organophosphorus poisons in the insect nervous system.

As shown in Fig. 1 (*c*, *d*) electrical nervous activity was found when recording electrodes were placed upon the abdominal nerve cord of the intact roach 6 hours after TEPP treatment. Yet no AChE activity was detected in ganglionic or peripheral nervous tissue. Nervous activity is clearly possible in nervous tissue of the roach despite inhibition of AChE. As shown in Table VII nervous activity was detectable in peripheral nerve fibers as well as in the abdominal nerve cord. Axonic nervous transmission occurred although AChE was known to be inhibited in these tissues (Table VII), thus confirming and extending Roeder's (19) observation that abdominal nerve cord axonic transmission was not inhibited in roaches when diisopropylfluorophosphonate (DFP) at 10^{-2} *M* was applied to the nerve cord. The unimpaired axonic nervous transmission in AChE-inhibited nervous tissue of the roach is contrary to the hypothesis of Nachmansohn (20) that AChE inhibition blocks nervous conduction in all types of nerves. The results for the cockroach are of particular interest since the nerve fibers are known to contain ACh (21).

The finding that axonic nervous transmission was possible in TEPP-treated roaches led to the supposition that much of the recorded nervous activity, an example of which is shown in Fig. 1 (*c*, *d*), may be the result of unimpaired afferent nervous activity. This possibility was strengthened when, as shown in Table VII, it was found that the electrical nervous activity of isolated nerve cords of TEPP-treated roaches was greatly diminished over that found in the intact roach. Isolation of the nerve cord from the roach removed peripheral sensory connections.

To test the assumption that afferent nerve fibers were responsible for a large part of the in vivo nervous activity, recording electrodes were placed upon various parts of the nervous system, and nerve connections were severed at points close to sense organs. The data given in Table VII show that electrical nervous activity in the abdominal nerve cord and fifth leg nerve was almost entirely abolished when afferent systems were eliminated. The absence of almost all of the previously recorded nervous activity showed that motor nerve

TABLE VII

Electrical nervous activity and acetylcholinesterase activity of nervous tissue of the roach at 6 hours after topical treatment with 5 μg TEPP per roach

Nervous tissue region	AChE activity in nervous tissue	Electrical activity in intact nervous tissue	Electrical activity in isolated nervous tissue
Spontaneous activity recorded from abdominal nerve cord	0	Some high frequency volleys of mixed nerve impulses followed by periods of quiescence	Little activity except for small fibers in the abdominal nerve cord
Cercal nerve – giant fiber synapse stimulated by puffs of air to the anal cerci	0 (sixth ganglion)	Facilitated response to first stimuli; followed by insensitivity	—
Afferent conduction in cercal nerve	0	Normal when anal cerci stimulated by air puffs	Normal when stimulated electrically
Ascending axonic conduction in the abdominal nerve cord	0 (abdominal nerve cord)	Giant fiber and smaller nerve fiber activity normal	Normal when stimulated electrically
Afferent conduction in fifth leg nerve caused by movement of spines	0	Normal	—
Motor conduction in fifth leg nerve induced by electrical stimulation	0	Normal	—
Spontaneous activity in thoracic connectives between third and second thoracic ganglia	0	Occasional high frequency volleys of mixed nerve impulses	Mostly absent
Section of the fifth leg nerve in femur	—	Afferent spontaneous activity abolished	—
Section of the abdominal nerve cord in front of sixth ganglion; recording electrodes on abdominal nerve cord	0	Spontaneous nervous activity mostly abolished	—
Application of 10^{-4} M TEPP in saline to sixth abdominal ganglion		Initial high frequency burst of nerve impulses	No stimulation of nervous activity

activity was reduced in TEPP-intoxicated roaches, providing an explanation of the inability of treated roaches to walk, for TEPP has no known effect on roach muscles (21).

The recorded afferent nervous activity given in Table VII led into the thoracic ganglia. This tissue was used for the determinations of ACh. As shown in Table IV no increase of ACh was found at 6 hours after TEPP treatment. It is also evident that AChE was completely inhibited in the thoracic nerve cord. In general, at 6 hours after TEPP treatment, the combined data for nervous activity, ACh and AChE show no correlation between these elements of the cholinergic system unless it be that the ACh released by nerve fibers has diffused away from the site of release into blood. The demonstration of ACh in roach blood (4) after TEPP treatment indicates that this is possible in the cockroach.

Roeder (19) and Yamasaki *et al.* (22) have shown that treatment of the sixth abdominal ganglion of the roach with anticholinesterases resulted in a block of the cercal nerve – giant fiber synapse, and inhibition of AChE. These experiments were carried out with nerve preparations where anticholinesterases were applied directly in saline to the ganglion. A different result was found, as shown in Fig. 1 (*e, f*), when the AChE of the sixth abdominal ganglion was inhibited after topical application of TEPP to the intact roach. Synaptic transmission was found to be unstable but not blocked at 6 hours after TEPP treatment. As shown in Fig. 1 (*e, f*), when sensillae of the anal cerci were stimulated by the technique of puffs of air (4) nerve impulses were transmitted through the sixth abdominal ganglion. Postsynaptic nervous activity was recorded between the fifth and sixth abdominal ganglion. The results for a normal roach are shown in Fig. 1 (*b*) and it is apparent that the synapse is abnormal in the TEPP-treated roach, for a repetitive response is shown in Fig. 1 (*e*) which was followed by a spontaneous discharge, Fig. 1 (*f*), and then block Fig. 1 (*g*) to a second stimulus. The synapse did not remain blocked as subsequent stimuli brought about results similar to those shown in Fig. 1 (*e, f*). In some experiments the spike heights appeared to diminish after successive stimulations. TEPP at 10^{-4} *M* in saline was then applied to an exposed ganglion. As shown in Table VII, the applied TEPP elicited a brief volley of nerve impulses. After this, ganglionic transmission was blocked, for it was impossible to record activity in response to stimulation of the sensillae. TEPP at the same concentration when applied to the sixth abdominal ganglion of the isolated nerve cord taken from TEPP-treated roaches failed to stimulate similar nerve impulses. The results of these experiments appear to show some form of synaptic response in the sixth abdominal ganglion in the intact roach, a response not found with the isolated preparation. The result found with the intact roach appears to be dependent upon an intact afferent nervous system and cannot be due to uninhibited AChE since, in all roaches examined, AChE was found to be completely inhibited. No proper explanation can be offered for these observations until more information is available about the type of synapse linking cercal nerve activity to nerve fibers in the abdominal nerve cord.

There are reasons for doubting the choice of selecting only the sixth abdominal ganglion for determining mechanisms of synaptic nervous transmission in the roach. The ganglion contains convergent type of synapses connecting a large number of cercal nerve fibers, having their origin in sensory nerve cells of hair-like structures on the anal cerci, to a few large nerve fibers which conduct stimuli applied to the hairs up the abdominal nerve cord into thoracic ganglion and possibly brain. The large fibers are known as giant nerve fibers and the nerve pathway described above is schematically illustrated by Roeder (23). Some of the giant fibers appear to synapse with motor fibers in the thoracic ganglia. In the intact roach puffs of air applied to the hair sense organs induces an alarm reaction causing the insect to jump forward. Eventually the roach ceases to respond to puffs of air, thus the insect shows a controlled behavior response to an external stimulus. Hughes and Colhoun (unpublished data) were unable to detect any modification of nervous transmission in the sixth abdominal ganglion, a result which suggests that modification or adaptation of the intact roach to air puff stimulus occurred in the brain or thoracic ganglia. The cercal nerve – giant fiber synapse may be a central sensory synapse. This synapse linking cercal nerve fiber with relatively few giant nerve fibers may be one of economy and efficiency thereby allowing maximum perception of stimulation to be channeled into a few fast-conducting nerve fibers. The sensory – motor nerve synapse appears to occur in the thoracic ganglion, which could be more aptly termed the giant nerve – motor nerve synapse. Thus mediation of nerve impulses in the sixth abdominal ganglion need not necessarily be similar to that occurring in the thoracic ganglion or brain. Furthermore the sixth abdominal convergent type synapse appears to be absent in other insects such as housefly. These facts appear to have been ignored when effects of poisons or drugs have been determined with the sixth abdominal ganglion preparation of the roach. After drug or insecticide treatment no attempts were made to measure disruption of nervous activity in the sixth abdominal ganglion of nerve fibers supplying motor activity to the anal cerci, the tip of the abdomen, or genitalia. These units may indeed represent the motor activity of the ganglion and in this respect they are opposite in direction to the cercal nerve – giant fiber system. Would transmission of nerve impulses in the two directionally opposed systems be dependent upon the same chemical mediator?

Discussion

The possibility that ALiE inhibition is a primary cause of organophosphorus poisoning in insects appears to be remote, for the roach, as well as the housefly (10), tolerates complete inhibition of ALiE without intoxication. The function of ALiE in the roach is obscure. The enzyme appears to be unassociated with mechanisms of nervous transmission in the roach, for no disruption of nervous activity was observed when ALiE was inhibited by TOCP treatment. ALiE is not specifically involved in nervous activity for the enzyme is found in non-nervous tissue.

Although not primarily a cause in organophosphorus poisoning in the roach,

inhibition of ALiE by TOCP resulted in potentiation of TEPP and parathion poisoning. This result suggests that more than one enzyme determines the effect of organophosphorus poisons in the roach. On the other hand, TOCP treatment of houseflies did not potentiate TEPP poisoning (10), thus showing a difference between two insect species. Furthermore, in the housefly, TOCP treatment resulted in some inhibition of AChE. AChE was not inhibited in the roach but in vitro the AChE's of roach and housefly were equally insensitive to TOCP. The in vivo inhibition of AChE by TOCP treatment in the housefly may be compared to that found in vertebrates, for Aldridge (13) has pointed out that, although pure TOCP per se is not an inhibitor of AChE, conversion to an anti-cholinesterase may occur in vivo. The apparent possibility of TOCP conversion occurring in the housefly shows another difference between the fly and the roach. Other enzyme differences are known for the two insect species, for Malathion was degraded in the housefly by phosphatases but in the roach by carboxyesterase and phosphatase action (2).

Inhibition of AChE appeared to be a primary lesion in TEPP poisoning. In roaches just prostrate by TEPP treatment, correlations were obtained between increase in the amount of ACh, AChE inhibition, and disruption of electrical nervous activity. Similar correlations were found in roaches treated first with TOCP and then with TEPP. Evidently ACh and AChE are involved in nervous activity in the roach, a finding contrary to that of Hopf and Taylor (25), who claimed normal nervous transmission in the locust when AChE was entirely inhibited. These workers believed that a phenylesterase is more important than AChE in organophosphate action, and presumably, nervous function. Colhoun (2) showed that nervous tissue of the roach contains the elements of the cholinergic system necessary for participation in the temporal and spatial events of nervous transmission and until an alternative enzyme and substrate are found, the cholinergic system must be held responsible for nervous transmission.

Examination of the degree of AChE inhibition and increases in ACh and the nervous activity at 6 hours after TEPP treatment revealed anomalies. At this time the amount of ACh was about the same or lower than that found in the nerve cord of an untreated roach. AChE was inhibited but nervous activity was not depressed although abnormal. The general level of electrical nervous activity was markedly lower than that found during the onset of TEPP poisoning, where significant increases in ACh were found. It is apparent that a high degree of nervous activity is associated with an increase in ACh. Disregarding the possibility that the residual nervous activity found at 6 hours was dependent upon a non-cholinergic system, these observations would have meaning if the ACh found in roach blood (4) had its origin in the central nerve cord. Coupled with a reduction in nervous activity a slow leakage of ACh from the nerve cord into blood would explain a relatively normal level of ACh in tissue where AChE was completely inhibited.

At 6 hours after treatment with TEPP the electrical nervous activity found with nervous tissue in the intact roach appeared to be afferent in origin, for

peripheral sectioning of nerve fibers eliminated most of the recorded nerve impulses. Hence afferent nervous systems were not inhibited in TEPP-intoxicated roaches, though AChE was inhibited in nervous tissue containing both sensory and motor nerve fibers. It was possible to stimulate motor nerve activity although in vivo the experimental evidence showed that spontaneous motor nerve activity was largely abolished. It is clearly evident that axonic nervous conduction in the roach was not dependent upon AChE activity. Unstable nervous synaptic transmission was found to occur in the sixth abdominal ganglion of intact TEPP-treated roaches but not in isolated nerve cords of these roaches. In each experiment AChE was inhibited. Since these results were found with the sixth abdominal ganglion, which may contain a particular type of central sensory synapse not found elsewhere in the roach or in other insects, other synapses should be examined to determine the suggested role of ACh and AChE in roach nervous function.

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NOTES

GROWTH OF EHRLICH ASCITES TUMOR CELLS IN TISSUE CULTURE

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In discussing the in vitro culture of Ehrlich ascites tumor cells Hull (1), Deschner and Allen (2), and Foley (3) have described the isolation of cell lines capable of growth on glass surfaces in serum-containing media. In this communication we wish to summarize some of our experiences in growing Ehrlich ascites cells in suspension culture as well as static culture.

Two methods were used in isolating the cell lines: cells removed from a mouse a week after injection with Ehrlich ascites tumor were added to 125 ml of Ziegler's medium (4) containing 10% (v/v) calf serum in a 250-ml flat-bottomed centrifuge bottle and agitated by a magnetic stirrer. The initial count of 2.2×10^5 cells per ml dropped to about 4.5×10^4 cells per ml after 5 days' incubation at 37°. Approximately 2.3×10^6 cells were added to a 1-liter Blake bottle and attached themselves to the glass surface. After several medium changes at weekly intervals, a complete cell sheet was obtained containing a mixture of rounded and fibroblastic forms. These cells were scraped from the glass surface and 1.2×10^6 cells were used to inoculate 30 ml of medium contained in a 1×6 in. screw-capped test tube. These tubes were placed on a Rollordrum apparatus (New Brunswick Scientific Co.) rotating at 60 r.p.m. and located in a 37° constant temperature room. Cell multiplication rates were low with generation times often approaching 3 to 4 days. After 3 weeks' incubation with weekly medium changes, the surviving cells were transferred back to the Blake bottles. They attached themselves to the glass surface and formed a complete sheet in a few days. These cells were used to initiate growth in suspension culture (with an initial count of 3×10^5 cells per ml) and continuous growth was obtained over a 10-month period. Generation times in suspension culture varying between 48 and 72 hours were usually obtained when the inoculum was of the order of 1×10^5 cells per ml. The cells in suspension culture were relatively large with many of the population having diameters over 25 microns, and the cell surfaces appeared to be sparsely covered with microfibrillar-like structures.

In other experiments successful growth of Ehrlich ascites tumor cells in tissue culture was also obtained by inoculation of 1-liter Blake bottles containing 75 ml of Eagle's medium (5) (supplemented with 10% (v/v) calf serum) with approximately 10 million cells taken from a tumor-bearing mouse 5 days after implantation. After 3 weeks' incubation at 37° with medium changes at 4- to 7-day intervals, a complete cell sheet was formed. These cells were scraped and the suspension used to inoculate other Blake bottles. The cell sheets formed in these secondary bottles after 6 days' incubation were used as inoculum for suspension cultures as described above. As the cells maintained in suspension culture produced significant amounts of lactic and

pyruvic acids (resulting in lowering of the pH of the medium from about pH 7.4 to pH 6.5 after 2 days' incubation), an attempt was made to control the pH of the medium. Several media were tested, and best results were obtained with Waymouth's medium (6) supplemented with 10% calf serum and 0.03% carboxymethylcellulose. Generation times varying between 24 and 48 hours were often observed. In addition to carrying the "standard Ehrlich ascites tumor cell line" (as used in the program of the Cancer Chemotherapy National Screening Center) in static culture (on glass surfaces) we have maintained in tissue culture cells taken from mice infected with "fluorouracil-resistant" Ehrlich ascites tumors. These tissue cultures have been subcultured many times at weekly intervals and have maintained a rapid growth rate in tissue culture with generation times of the order of 2 days.

Deschner and Allen (2) mentioned that a cell line derived from Ehrlich ascites cells taken from a tumor-bearing mouse and maintained in glass-surface tissue culture no longer formed tumor cells when injected into mice. We have found that no tumors were formed when up to 1000 cells taken from our suspension cultures were injected into mice. This may indicate that some selection of the original tumor-cell population occurred when the cells were grown on the glass surfaces prior to initiation of suspension culture.

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EVALUATION OF PROTEIN QUALITY IN MIXED FOODS

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In previous studies on evaluation of protein quality, McLaughlan *et al.* (1) developed a simplified chemical score, based on the lysine, methionine, and cystine content of individual foods. A good correlation was observed between simplified chemical score and protein efficiency ratio (P.E.R.) values for a considerable number of foods. In subsequent studies, Morrison and Campbell (2) found that P.E.R. values for bread and flour diets supplemented with fish flour were a direct function of the lysine content of the protein. In an extension of these studies, it has been found that measurement of the lysine content also provides an accurate evaluation of the nutritional value of the protein in a variety of mixed foods.

In the mixtures studied, half of the protein was supplied by bread, and the other half by bread, casein, whole egg powder, fish flour, cooked beans, protein

cereal plus milk (1:4), cheddar cheese, or oatmeal. The mixtures were incorporated into the protein-free basal diet of Chapman *et al.* (3) at the 10% protein level and fed to groups of male weanling rats for 4 weeks. P.E.R. values (g gain/g protein consumed) were then calculated. Lysine, methionine, and cystine were determined by previously described procedures (1, 4) using 8-hour acid hydrolyzates.

Data on the relationship between P.E.R. and lysine content of the protein are shown graphically in Fig. 1. The almost perfect correlation observed ($R=0.99$) indicates that for the variety of mixed proteins studied, biological

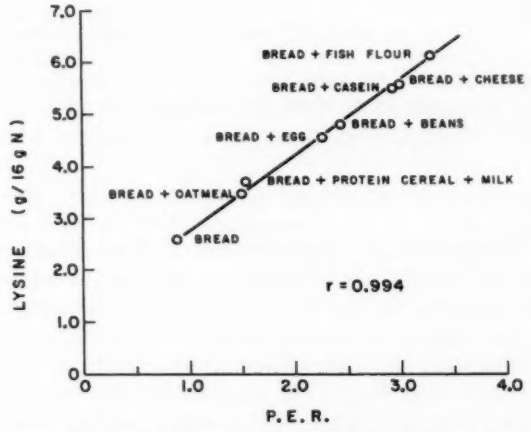

FIG. 1. Correlation between lysine content and P.E.R. for several mixtures of foods.

value, as measured by P.E.R., was directly dependent upon the lysine content of the protein. This relationship held, without exception, for P.E.R. values varying from 0.9 (bread alone) to 3.32 (bread plus fish flour). A good correlation between P.E.R. and methionine plus cystine content of the protein was also observed for mixtures of bread plus casein, cheese, fish flour, or beans, although in no case was the correlation better than that between P.E.R. and lysine. The results suggest that in these mixtures of foods, lysine and cystine plus methionine were approximately equally limiting. P.E.R. values for bread plus egg, protein cereal and milk, or oatmeal, or for bread alone, however, did not correlate closely with methionine plus cystine content. Reexamination of simplified chemical score data for mixtures of oat, wheat, and corn cereals plus milk (1) showed that the P.E.R. of these mixed proteins could be estimated as accurately from the lysine content as from the methionine plus cystine content.

It may be concluded, therefore, that for mixtures of foods in which cereal products contribute approximately half or more of the protein, the lysine content is a reliable guide to the nutritional value of the protein mixture.

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MODIFICATIONS OF THE ASSAY FOR SMALL AMOUNTS OF ANTIDIURETIC HORMONE*

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A method has been described from this laboratory for the determination of minute amounts of antidiuretic hormone activity by bio-assay in the conscious rat (1). In this procedure stomach and bladder cannulae were inserted on the previous day, and the external jugular vein was cannulated under very light ether anaesthesia on the day of the assay. On recovery from the operation, the rats were kept in a tranquil though conscious state by the use of perphenazine. This assay has worked well, but the delay in onset of maximal rates of diuresis following the jugular cannulation has limited the effective duration of the working day. This inconvenience has been overcome by the following modification of procedure.

All cannulations, including that of the jugular vein, are done before the assay. The polyethylene cannula (60 gauge) in the jugular vein is brought out through a stab wound between the ears, as described by Ginsburg and Heller (2). It is filled with heparinized saline, and a full-length obturator of No. 10-gauge polyethylene tubing is inserted and sealed. The rat is given a dose of perphenazine 1.0 mg/100 g of body weight by intraperitoneal injection, and placed in a cylindrical confining cage of wire mesh, which prevents the rat from pawing or chewing the cannulae. Free access to food and water is provided.

The next morning, the obturator is removed and the various cannulae are connected as described previously (1). Hydration is immediately carried out via the gastric cannula but with 1% ethanol rather than the dilute saline formerly used. The alcohol maintains the rat in a quiet but conscious state, replacing the effect of the perphenazine which is by now worn off. Mirsky and his colleagues also have found that the use of dilute ethanol provides adequate sedation in rats prepared in this manner (3). While the box in which

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the rat is placed helps to keep its immediate environment relatively constant, more uniform results are obtained if the room temperature is kept between 72 and 78° F. The measurements and calculations are the same as those previously described.

As a result of these changes, each rat can be used continuously for 6–8 hours. This increases substantially the number of samples that may be assayed on each rat in a normal working day with no loss of statistical precision.

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A COMPARISON OF THE EFFECTIVENESS OF INTRAVENOUS, AS OPPOSED TO SUBCUTANEOUS, INJECTION OF PROGESTERONE FOR THE INDUCTION OF ESTROUS BEHAVIOR IN THE RAT

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It has been shown that the administration of estrogen followed in proper sequence by a suitable dosage of progesterone is more effective than estrogen alone for inducing behavioral estrus in the spayed guinea pig (1), rat (2), mouse (3), and hamster (4). Behavioral estrus induced by subcutaneous injection of estrogen in oil, followed 48 hours later by progesterone similarly administered, has a latency of from 2 to 4 hours after the progesterone injection (2). Kent and Liberman (5) have shown for the hamster that, after suitable priming by estrogen, a small dose of progesterone in oil injected directly into the third ventricle of the brain induces estrus in 10 to 20 minutes.

Several investigators have shown that progesterone is rapidly destroyed in the body (6, 7). Now, if progesterone were injected directly into the blood stream, it should reach the active sites most rapidly and it seems reasonable to expect a decrease both in latency of action and in the dose required to elicit a response. This hypothesis was tested in the following experiments, the easily characterized reaction of lordosis being taken as the biological assay of a positive response.

Materials and Methods

All rats used were adult virgins of the Harvard strain. The animals were castrated 2 to 3 weeks before receiving the initial estrogen injection. The estrogen priming regimen consisted of 10 μg estradiol in sesame oil daily for 3 days, injected subcutaneously.

Twenty-four hours after the final estradiol injection the animals were tested for mating responses. Each female was placed in a mating cage with two active males for a 10-minute period. Any female showing a lordosis

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response at this time was discarded. The remaining animals were, one at a time, rolled into a towel and the towel firmly pinned about the rat so that one hind leg protruded. Varying amounts of progesterone in 0.05 ml propylene glycol was injected into the saphenous vein following the method of Everett and Sawyer (8). At completion of the injection the rat was released from the towel and placed in a mating cage, and the time to lordosis recorded.

Results and Discussion

When estrogen-primed rats were injected intravenously with from 25 to 400 μg of progesterone all but two of 24 animals showed behavioral estrus in from 2.5 to 10 minutes after the progesterone injection. Ten minutes was taken as an arbitrary limit during which a lordosis response must have occurred for a test to be considered positive. Figure 1 shows the percentage of

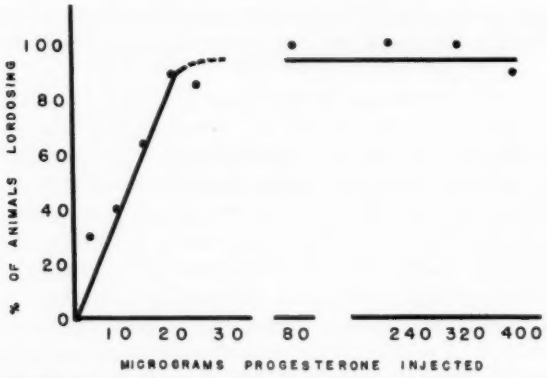

FIG. 1. Percentage of castrate female rats showing a lordosis response within 10 minutes of an intravenous injection of progesterone.

positive responses at various progesterone levels. It is immediately apparent that after 20 to 400 μg of progesterone 90 to 100% of the animals show the lordosis response within 10 minutes of the injection. At this point the curve appears to break sharply, and for all lesser values shows a linear relationship between number of animals lordosing and amount of progesterone injected.

On subcutaneous injection of 400 μg progesterone in propylene glycol into estrogen-primed rats the latency before the animals would lordose was 120 to 400 minutes. After subcutaneous injection of 25 μg progesterone none of 10 animals tested showed a lordosis response. Intravenous injection of propylene glycol alone did not produce estrous behavior.

The short latent period to appearance of behavioral estrus after intravenous injection of progesterone supports the findings of Kent and Liberman (5) for the hamster when progesterone was injected directly into the third ventricle. From the experiments reported here the following significant conclusions may be drawn. (*a*) The intravenous injection of progesterone in the rat decreased the dose required to stimulate behavioral estrus to 1/60th of that required by

the subcutaneous route. This difference may be due in part to the rapid turnover rate of progesterone in the body (6); it may be presumed that the amount of hormone reaching the brain when administered subcutaneously must be considerably less than when the same dose is injected into the saphenous vein. (*b*) The latent period of the response to progesterone is relatively short, 2½ to 10 minutes as compared with 2 to 4 hours after subcutaneous injection. Intravenous experiments conducted by Zander (7) with humans indicate that, if progesterone itself reacts at an active center, its reaction time must be very short due to the rapid disappearance of the hormone from the blood.

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BIOLOGICAL CONVERSION OF 4-NITROBIPHENYL TO AN ACTIVE CARCINOGEN*

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Because of its close structural relationship to 4-aminobiphenyl, it was thought that the carcinogenicity of 4-nitrobiphenyl, reported recently by Deichmann and co-workers (1), was due to the fact that it was reduced in vivo to the amino derivative, which is an active carcinogen (2, 3, 4, 5). The experiments described here were aimed at the identification of 4-aminobiphenyl and eventually other products of transformation.

Sprague–Dawley rats, weighing approximately 150 grams, each received one oral dose of 16 mg of 4-nitrobiphenyl dissolved in 1 ml of corn oil. Urine was collected for 5 days and spotted, along with standard substances, on Whatman paper No. 1 and the chromatograms run in five different solvent systems. Detection of the metabolites was made with 10 different reagents (6), and the chromatograms viewed under ultraviolet light (2537 Å and 3660 Å).

As shown on the chromatogram (Fig. 1), there is evidence of formation of 4-aminobiphenyl and of 4-amino-3-biphenylyl hydrogen sulphate. Although

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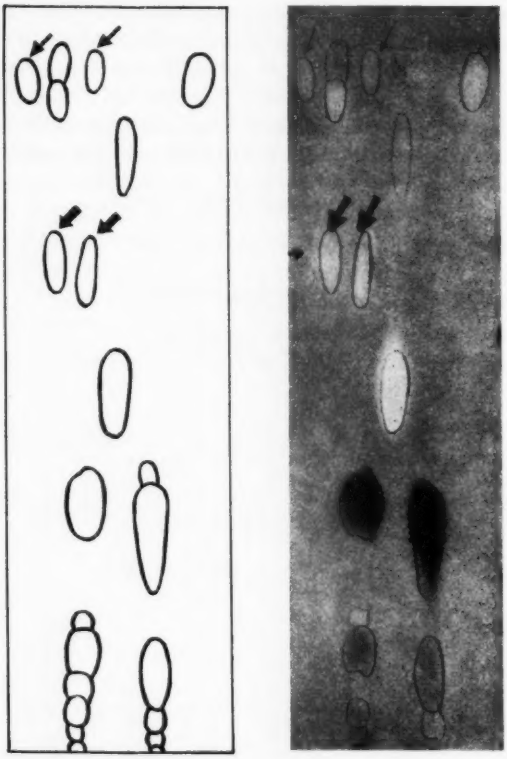

FIG. 1. Chromatogram run in butanol – acetic acid – water v/v (4:1:5). Descending method. Time: 15 hours. This photograph (color) was taken under ultraviolet light (3660 Å) after the paper was sprayed with Ehrlich reagent (6). Several spots which were detected under ultraviolet before the spray did not show up after it. Reading of numbers is to start from the solvent front.

a. (1) 4-Aminobiphenyl (thin arrow).
b. (1) 3-Hydroxy-4-aminobiphenyl. The top part of this spot is an artifact (dark).
(2) Ester sulphate of *b* (1) (thick arrow).
c. Urine of rats fed with 4-nitrobiphenyl.
(1) 4-Aminobiphenyl (thin arrow).
(2) Ester sulphate of *b* (1) (thick arrow).
(3) Urea.
d. (1) 4-Amino-4′-hydroxybiphenyl.
(2) 4-Aminobiphenylylsulphamic acid (K salt).
e. Urine of control rats fed with corn oil.
(1) Urea.
f. (1) 3-Hydroxy-4-nitrobiphenyl.
4-Nitrobiphenyl, which was spotted along with *f* (1), did not show up after the spray.

the latter compound was the only metabolite isolated as yet (Fig. 2), other products of transformation have, however, been detected by paper chromatography of the urine collected during the first 5 days.

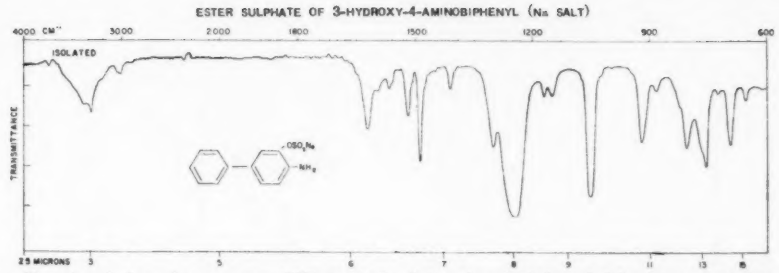

FIG. 2. Infrared spectrum (KBr pellet) of 4-amino-3-biphenylyl hydrogen sulphate (Na salt) isolated from the urine of rats fed with 4-nitrobiphenyl. 4-Amino-3-biphenylyl hydrogen sulphuric acid was isolated by several extractions of acidified urine with *n*-butanol and converted to the sodium salt according to Boyland and Sims (9). After acid hydrolysis of this metabolite, 3-hydroxy-4-aminobiphenyl was obtained (m.p. and mixed m.p. = 183°; reported (9): 182–184°).

It was not possible to identify free 3-hydroxy-4-aminobiphenyl in freshly obtained urine. After administration of 4-aminobiphenyl (20 mg/kg) and its 3-hydroxy-derivative (15 mg/kg) to two other groups of rats, we failed to identify free hydroxylated derivatives, but we isolated, however, a rather high amount of 4-amino-3-biphenylyl hydrogen sulphate (respectively 10 and 12% excreted in 20 days), which supports the idea that the intermediary metabolites are conjugated at a very fast rate. Similar results have been obtained by Bradshaw (8), who was unable to identify free hydroxy-amines in freshly voided urine of dogs dosed with 4-aminobiphenyl.

A study of the comparative metabolic fate of these and other biphenyl derivatives in various animal species is in progress in our laboratory. Pending the publication of a more detailed report (7), it is, however, possible to postulate, from the results recently obtained, the following metabolic scheme (Fig. 3).

Many thanks are due to Mr. John W. Sinclair for his assistance.

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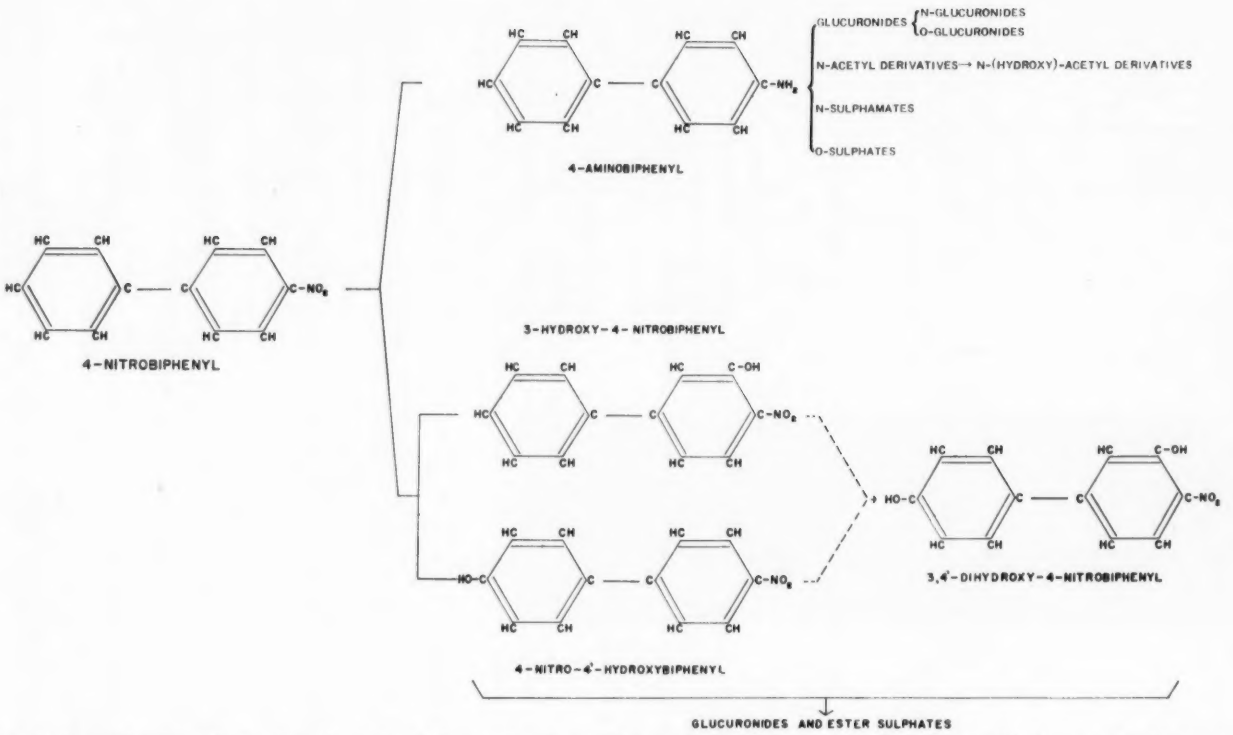

FIG. 3. A postulated metabolic scheme for 4-nitrobiphenyl. Another possible metabolite, not shown in this scheme, is 2′-hydroxy-4-nitrobiphenyl.

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